Skeletal muscle microRNAs in human cancer cachexia and Type 2 diabetes

Robin A McGregor

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School of Engineering and Physical Sciences

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ABSTRACT

MicroRNAs are powerful post-transcriptional regulators of gene expression and are biomarkers of chronic diseases such as cancer. This thesis explores the role of microRNAs in human cancer cachexia and Type 2 diabetes. MicroRNA expression was measured in skeletal muscle biopsies using RT-qPCR. In pancreatic cancer cachexia patients, expression of microRNA-1, microRNA-133a, microRNA-133b and microRNA-206 was negatively related to weight loss. In Type 2 diabetes skeletal muscle, microRNA-133a and microRNA-206 expression was down-regulated, but there was no evidence of altered microRNA transcription or processing and target expression was unchanged. Importantly, microRNA-133a expression predicted fasting glucose, glucose tolerance and insulin resistance, and therefore may be a biomarker of Type 2 diabetes. Experimental validation of microRNA arrays was unsuccessful in identifying further novel cancer cachexia and Type 2 diabetes microRNA biomarkers. MicroRNA knockdown validated CDC42 and PTBP1 as microRNA-133a targets in myoblasts. In addition, muscle microRNA expression may be regulated by insulin and TNFα. In conclusion, microRNA-133a may be a skeletal muscle biomarker of Type 2 diabetes and cancer cachexia, microRNA-133a responds to extracellular insulin and TNFα, but it remains to be established whether microRNA-133a contributes to cancer cachexia or Type 2 diabetes pathogenesis.

DEDICATION

I dedicate my PhD thesis to mum and dad.

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DECLARATION STATEMENT

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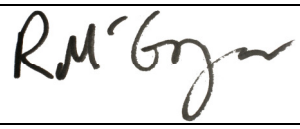
Signature of Candidate*:*		Date:	**1st December 2009**

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List of Abbreviations

3'UTR	3' Untranslated region
ADA	American Diabetes Association
AGO	Argonaute
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ASO	Antisense oligonucleotide
ATP	Adenosine-5'-triphosphate
BMI	Body mass index
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
CDC42	Cell division control protein 42
cDNA	Complementary DNA
CRP	C-reactive protein
CT	Cycle threshold
CV	Coefficient variation
DEPC	Diethylpyrocarbonate
DEXA	Dual energy X-ray absorptiometry
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
FDR	False discovery rate
GK	Goto-Kakizaki
GO	Gene ontology
hbA1c	Glycated haemoglobin
HC	Healthy control
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HOMA [IR]	Homeostasis model assessment of insulin resistance
ICU	Intensive care unit
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IL	Interleukin
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDL	Low-density lipoprotein
LNA	Locked nucleic acid
MAMC	Mid-arm muscle circumference
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
MOPS	Morpholino propanesulfonic acid
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIF	Proteolysis inducing factor
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTBP1	Polypyrimidine tract binding protein 1
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
RIN	RNA integrity number
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT-qPCR	Real time quantitative polymerase chain reaction
S6K	S6 protein kinase
SAM	Statistical analysis of microarrays
SDS	Sodium dodecyl sulphate
shRNAi	Short hairpin RNA inference
SILAC	Stable isotope labelling amino acids in cell culture
SMEK2	Suppressor of MEK
SRF	Serum response factor
SSPE	Sodium Chloride-Sodium Phosphate-EDTA buffer
T2D	Type 2 diabetes
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TGIF2	TGFb-induced factor 2
TNF	Tumour necrosis factor
VAMP	Vesicle associate membrane
VO_{2MAX}	Maximum oxygen uptake

List of Abstracts and Publications

McGregor, R.A., Keller, P., Nielsen, A.R., Sewell, D., Fischer, C.P., Timmons, J.A., and Pedersen, B.K. (2008). Down-regulation of microRNA expression in Type 2 diabetes implicates non-coding RNA in insulin action. Abstract presented at the *Keystone MicroRNA, and Non-Coding RNA Meeting*, Whistler, Canada.

Chapter 1 - Introduction

Skeletal muscle phenotype and function is determined by the transcription and translation of thousands of genes. Muscle dysfunction is a key player in the pathophysiology of Type 2 diabetes (Muoio & Newgard, 2008; Cohen, 2006; Khan & Pessin, 2002; Schinner et al. 2005; Zeggini et al. 2008) and wasting conditions such as cancer cachexia (Fearon, 1992; Fearon et al. 2006; Stewart et al. 2006; Tan & Fearon, 2008; Stephens et al. 2008), which are characterised by profound changes in skeletal muscle phenotype. It has been suggested common transcriptional changes in skeletal muscle gene expression may occur in many systemic diseases such as diabetes, cancer, AIDS and renal failure, but expression of over 90% of genes appear not to change (Lecker et al. 2004). Therefore many genes in these systemic diseases may be regulated post-transcriptionally or alternatively changes in 10% of genes determine skeletal muscle phenotype in these systemic diseases.

The human genome contains many genes that do not code for proteins, including genes coding for microRNAs, which were first identified almost a decade ago (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee & Ambros, 2001). MicroRNAs may regulate over one third of protein coding genes post-transcriptionally. Indeed microRNAs have challenged our traditional understanding of gene regulation over the past decade (Bartel, 2004; Bartel, 2009; Ambros, 2004; Kim et al. 2009).

1.1. MicroRNAs

MicroRNAs (microRNA) consist of 19-22 nucleotides, which do not code for proteins, but regulate gene expression of protein-coding transcripts (Bartel, 2004; Bartel, 2009). Understanding of how microRNAs regulate gene expression has advanced significantly over the last decade (Lagos-Quintana et al. 2001; Ambros, 2004; Bartel, 2004; Bushati & Cohen, 2007; Bartel, 2009). The most clearly defined mechanism so far involves suppression of gene expression post-transcriptionally (Jackson & Standart, 2007). MicroRNAs can suppress translation of transcripts by base pairing to the 3' untranslated regions (3'UTRs) of protein coding genes and each microRNA is predicted to target >200 gene transcripts (Lall et al. 2006; John et al. 2004; Lewis et al. 2003). Overexpression and knockdown of microRNAs have been demonstrated to profoundly affect cell phenotype (Bushati & Cohen, 2007; Ambros, 2004). There is now evidence suggesting microRNAs play a fundamental role in many biological pathways, including

adipocyte and skeletal muscle differentiation (Chen et al. 2006; Nakajima et al. 2006; Boutz et al. 2007; Yuasa et al. 2008; McCarthy et al. 2007; Kim et al. 2006; Rosenberg et al. 2006). Some studies suggest that microRNAs may also play a role in maintaining amino acid, lipid and carbohydrate metabolism (Bushati & Cohen, 2007; Ambros, 2004). This has led to the intriguing possibility that microRNAs are involved in disease, with the majority of studies focused on cancer (Blenkiron et al. 2007; Lu et al. 2005; Dixon-McIver et al. 2008; Szafranska et al. 2008).

1.2. MicroRNAs are biomarkers of many chronic diseases

The discovery of microRNAs has provided exciting new possibilities for the identification of disease biomarkers, thus potentially facilitating earlier disease detection and development of new therapeutic agents. There have been many reviews suggesting microRNAs may lead to aberrant gene expression in major diseases involving skeletal muscle such as Type 2 diabetes (Gauthier & Wollheim, 2006; Hennessy & O'Driscoll, 2008; Poy et al. 2007; Tang et al. 2008), cardiac hypertrophy (Carè et al. 2007), muscular dystrophy (van Rooij et al. 2008; Chen et al. 2009; Yang & Wu, 2007) and HIV/AIDS (Bushati & Cohen, 2007; Couzin, 2008; Perera & Ray, 2007), but there have been few clinical studies in patients.

1.3. MicroRNA changes in skeletal muscle

There is evidence to suggest microRNAs play an important role in skeletal muscle (van Rooij et al. 2008; Chen et al. 2006; Chen et al. 2009; McCarthy & Esser, 2007; McCarthy et al. 2007). Firstly, during differentiation there is a significant increase in expression of muscle-specific microRNAs including miR-1, miR-133a and miR-206 (Chen et al. 2006). Secondly, microRNAs appear to be modulated during skeletal muscle hypertrophy in mice and humans (Drummond et al. 2008; McCarthy & Esser, 2007). Thirdly, microRNAs are modulated during skeletal muscle atrophy and wasting, for example in response to hind-limb unloading, space flight and muscular dystrophy (Allen et al. 2009; Eisenberg et al. 2007; McCarthy et al. 2007; Rosenberg et al. 2006). Fourthly, microRNAs in skeletal muscle respond to endurance training in mice and humans (Keller et al. 2007; Safdar et al. 2009). Taken together these studies suggest microRNAs may play a role in skeletal muscle dysfunction in chronic diseases such as Type 2 diabetes and cancer (Couzin, 2008; Hennessy & O'Driscoll, 2008). However, there have been no previous studies on microRNA function in skeletal muscle in cancer or Type 2 diabetes patients.

1.4. Cancer cachexia

Cachexia is a complex syndrome, which is characterised by progressive loss of skeletal muscle and is reportedly a significant contributing factor to mortality rates in cancer patients (Fearon, 1992). Skeletal muscle wasting has major implications for quality of life and physical function (Stewart et al. 2006). Some degree of weight loss is experienced by about 50% of all cancer patients (DeWys, 1985). There is no clearly defined physiological profile of cancer cachexia patients. Studies have considered many factors including alterations in protein, glucose and fat metabolism; circulating cytokines; tumour derived factors; production of acute phase reactants (Skipworth et al. 2007; Melstrom et al. 2007; Baracos, 2006; Laviano et al. 2005; Rubin, 2003). Importantly, there still appears to be no reliable biomarker of cancer cachexia (Tan & Fearon, 2008), thus more research is warranted to identify biomarkers, which can detect cachexia in its early stages.

It is clear microRNAs are key players in skeletal muscle differentiation and there are indications that microRNAs play a role in skeletal muscle remodelling, growth and atrophy (Allen et al. 2009; Eisenberg et al. 2007; Keller et al. 2007; McCarthy et al. 2007; Rosenberg et al. 2006; Safdar et al. 2009). Therefore, it is plausible that microRNA expression in muscle may play a role in cancer cachexia development or could be a biomarker of cancer cachexia progression. Previous findings on atrophy-associated microRNAs have been mixed due to the use of different atrophy models (Eisenberg et al. 2007; McCarthy et al. 2007; Rosenberg et al. 2006; Safdar et al. 2009). As yet there have been no studies on microRNA expression in skeletal muscle of cancer cachexia patients.

1.5. Type 2 diabetes

Skeletal muscle sensitivity to insulin is essential to facilitate glucose disposal in response to feeding. Impairment of skeletal muscle insulin sensitivity is a key factor in the development of Type 2 diabetes. However, the mechanisms responsible for the development of insulin resistance and the progression to Type 2 diabetes are still not fully understood (Muoio & Newgard, 2008; Shoelson et al. 2006). It is known skeletal muscle is the main site for whole-body glucose disposal, and insulin sensitivity is a key determinant of muscle glucose uptake. Decreased insulin sensitivity leads to impaired glucose tolerance and elevated plasma glucose concentrations despite normal β-cell

function. Development of Type 2 diabetes is characterised by loss of glycaemic control and eventual β-cell failure (Muoio & Newgard, 2008; Shoelson et al. 2006).

To date microarray studies on Type 2 diabetes have been unable to produce convincing evidence of a global change in gene expression profile despite the development of an insulin resistant muscle phenotype (Frederiksen et al. 2008; Mootha et al. 2003; Patti et al. 2003; J. Timmons et al. personal communication). This raises the intriguing possibility that a post-transcriptional mechanism may play a role. In diabetic rats, microRNA array studies have revealed several candidate Type 2 diabetes microRNAs, but there is a lack of consistency between studies (He et al. 2007; Huang et al. 2009). Importantly, there have been no clinical studies examining changes in microRNA expression in skeletal muscle of Type 2 diabetes patients.

1.6. Approach

This thesis will focus on determining the role of microRNAs in two chronic diseases affecting human skeletal muscle: Type 2 diabetes characterised by skeletal muscle insulin resistance (Muoio & Newgard, 2008; Shoelson et al. 2006) and cancer cachexia characterised by skeletal muscle wasting (Tisdale, 2005). The most robust, sensitive and practical method for profiling microRNA expression currently appears to be using real-time quantitative PCR (RT-qPCR; Ach et al. 2008; Chen et al. 2005), so RT-qPCR was the main methodology employed for detection of microRNAs.

In Chapter 3, expression of the muscle specific microRNAs, miR-1, miR-133a, miR-133b and miR-206 was profiled in human skeletal muscle from cancer patients to determine whether these microRNAs may be early biomarkers of cachexia. In addition, the inflammation-associated miR-21 was also measured as a potential mediator of protein degradation pathways in cachexia. MicroRNA array data from cancer-cachexia patients (I. Gallagher, personal communication) was subject to experimental validation using RT-qPCR to help identify new candidate microRNA biomarkers in cancer cachexia. Bioinformatics was used to examine the biological processes and disease associated pathways targeted by the candidate microRNA biomarkers identified. Finally, unpublished microarray data (I. Gallagher, personal communication) from cancer cachexia patients was used to analyse microRNA target expression to determine whether there was evidence of microRNA action on targets *in-vivo*.

In Chapter 4 the thesis focuses on examining the involvement of microRNAs in Type 2 diabetes. Expression of muscle-specific microRNAs, miR-1, miR-133a, miR-133b and miR-206 was profiled in patients with varying degrees of insulin resistance and glucose tolerance. Then, microRNA transcription and processing were measured to determine whether there were any alterations, which may help explain changes in mature microRNA expression in Type 2 diabetes. To establish whether microRNAs may play a role in developing insulin resistance and Type 2 diabetes, multiple regression was used to determine whether skeletal muscle microRNA expression could predict fasting glucose, glucose tolerance and insulin resistance in patients. A bioinformatics approach was used to determine which biological and cellular functions may be affected by microRNA changes in Type 2 diabetes and whether these could plausibly contribute to insulin resistance and Type 2 diabetes pathogenesis. Microarray data from Type 2 diabetes patients (n = 118; J. Timmons, personal communication) was used to determine whether there was evidence of microRNA action on targets *in-vivo*. Finally, microRNA array data from Type 2 diabetes patients (I. Gallagher, personal communication) was subject to experimental validation using RT-qPCR to help identify new candidate microRNA biomarkers in Type 2 diabetes.

In Chapter 5 a mechanistic approach was taken to validate specific targets of microRNAs and to examine possible regulators of microRNAs in Type 2 diabetes and cancer cachexia. MicroRNA knockdown experiments were conducted in muscle cells to determine the effect on microRNA target proteins including CDC42 and PTBP1. To investigate whether extracellular factors associated with both Type 2 diabetes and cancer cachexia could be partly responsible for the changes in microRNA expression observed *in-vivo*, microRNA expression was measured in muscle cells treated with insulin or TNFα.

This thesis demonstrates microRNAs may be involved in the development of both Type 2 diabetes and cancer cachexia and furthermore microRNAs may provide early biomarkers of skeletal muscle changes in these chronic diseases therefore facilitating earlier intervention. This thesis is unique as it presents the first evidence of microRNA changes in skeletal muscle from Type 2 diabetes and cancer cachexia patients. The microRNAs identified in this thesis may provide important early biomarkers of Type 2 diabetes and cancer cachexia, thus potentially facilitating earlier intervention and treatment before further complications and decreases in quality of life occur.

Chapter 2 - Literature review

MicroRNAs are a class of non-coding RNAs in the human genome, which do not code for proteins (Bartel, 2004). Many thousands of genes are targeted by microRNAs, which act post-transcriptionally to regulate cellular protein levels (Bartel, 2009). In less than a decade since the discovery of microRNAs they have been established as global regulators of multiple cellular processes (Bartel, 2009; Ambros, 2004; Bushati & Cohen, 2007). Distinct microRNA signatures are associated with a wide-range of chronic diseases including cancers, cardiovascular disease, HIV/AIDS and muscle wasting (Couzin, 2008; Chen et al. 2009; van Rooij et al. 2008; Eisenberg et al. 2007). In 2008 alone over 1300 microRNA studies were published. Due to the rapidly expanding interest in microRNA research our understanding of microRNA biogenesis and function is constantly evolving with new research challenging existing ideas (Bartel & Chen, 2004; Kim et al. 2009).

2.1. Discovery of microRNAs

Several studies were published simultaneously that reported the identification of a large class of small 20-23 nucleotide RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee & Ambros, 2001). These microRNAs were first identified by cloning in *C.elegans* and Drosophila, but remarkably many appeared to be highly conserved across species. A group of microRNAs found in the *Drosophila melanogastor* genome was also found in human HeLa cells. Northern blotting of these microRNAs revealed some were present only during embryogenesis and not at later stages of development, whereas some microRNAs were present at all stages of development. For example, miR-1 was expressed in the musculature of adult flies but was undetectable in HeLa cells (Lagos-Quintana et al. 2001). This led to the proposal that microRNAs may play a regulatory role in tissue specification. When some of these microRNAs were verified using Northern blot two additional signals were detected around 70 nt and 1000 nt in the case of miR-1 (Lagos-Quintana et al. 2001). These were subsequently classified as precursor microRNA (pre-microRNA) and primary microRNA (pri-microRNA) respectively (Griffiths-Jones et al. 2006). At this point it was not clear why microRNAs were processed or how they were processed. But it was clear microRNAs must be transcribed as much longer primary transcripts (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee & Ambros, 2001).

2.2. MicroRNA transcription, biogenesis and processing

Genomic analysis of these new novel microRNAs revealed all were flanked by sequences that could form ~70 nt stem-loop structures (Lagos-Quintana et al. 2001). Some of these microRNA stem-loop sequences aligned to longer mammalian ESTs suggesting that 20-23 nt small RNAs may be derived from longer transcripts. The genomic location of microRNAs suggested they were transcribed in diverse ways. Approximately, 30% are located in intronic regions and many are located in intergenic regions indicating they are transcribed from their own promoter (Lagos-Quintana et al. 2001). Intronic regions are located between exons of protein coding genes and intergenic regions are located outside of protein coding sequences. At this time little was known of how microRNAs were processed; comparison of sequencing data with genomic and EST data suggested several steps were involved.

2.2.1 MicroRNA transcription

Experiments involving chromatin immunoprecipitation analysis demonstrated that the polymerase poll II can be physically associated with a microRNA gene (Lee et al. 2004). Inhibition of poll II activity by treatment of human cells with alpha-amanitin reduces pri-microRNA concentration, which suggested poll II could be the main polymerase for microRNA gene transcription (Lee et al. 2004). A later study found poll III could be required for transcription of around 50 microRNA genes (Borchert et al. 2006). Chromatin immunoprecipitation data surrounding microRNA genes found 20% of intergenic microRNAs have there own promoter, suggesting intergenic microRNAs can be transcribed as independent transcriptional units (Corcoran et al. 2009). In contrast, intronic microRNAs appear to be transcribed in unison with their host gene (Rodriguez et al. 2004), based primarily on the observation that intronic microRNA expression is often correlated with predicted host-gene expression (Wang & Li, 2009; Baskerville & Bartel, 2005).

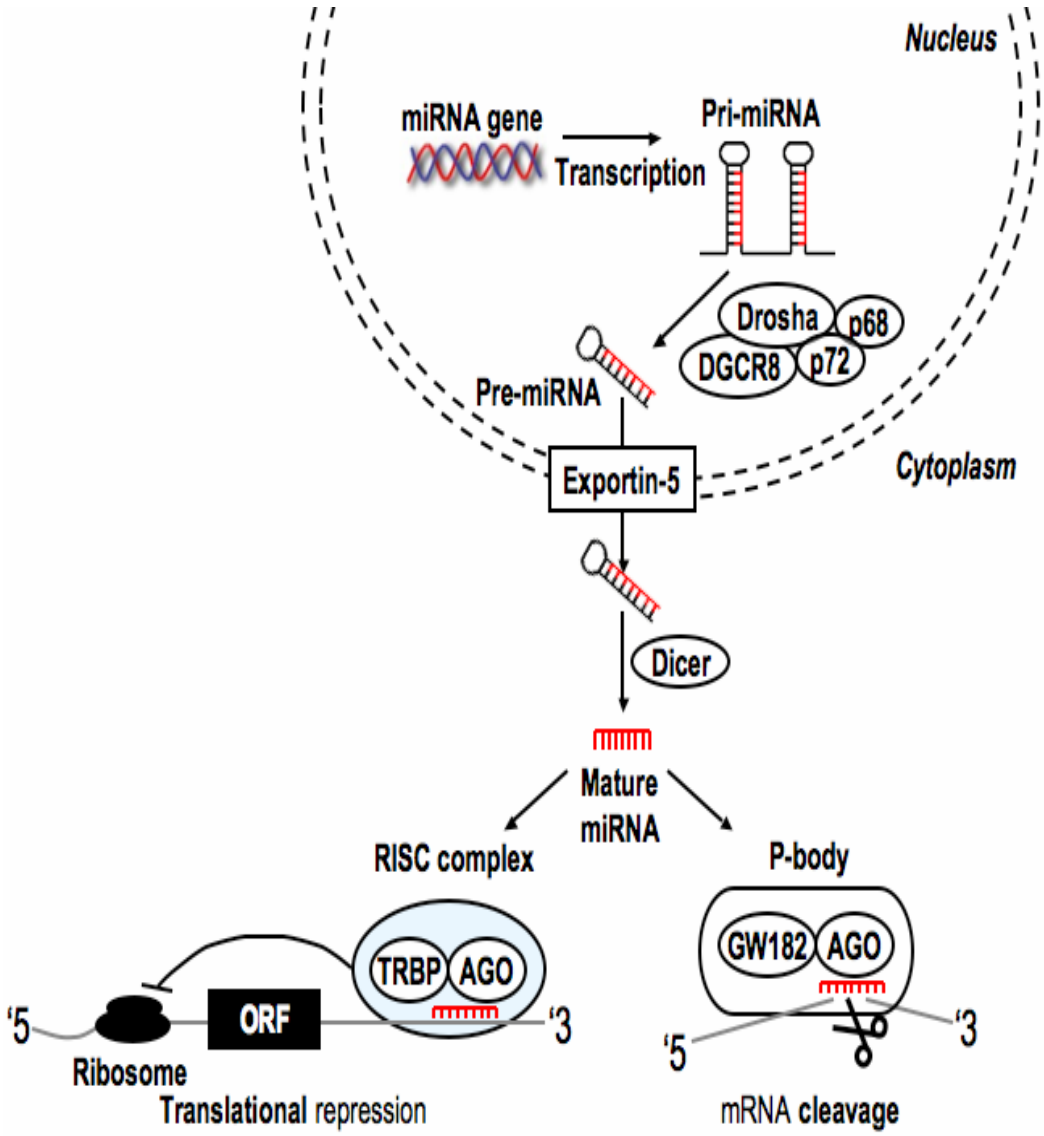


Figure 2-1. MicroRNA biogenesis and processing pathway.

MicroRNAs are transcribed as long pri-microRNA transcripts. Pri-microRNAs are cleaved by a microprocessor complex consisting of DICER/DGCR8/p68/p72. Pre-microRNA are exported to the cytoplasm via EXPORTIN5. Mature microRNAs are the result of pre-microRNA cleavage by DICER. Mature microRNAs are incorporated into an RNA-induced silencing complex (RISC) and bind AGO/TRBP or associate with P-bodies leading to translational repression or mRNA cleavage (Adapted from Kim et al. 2009)

Currently, few primary microRNA transcripts have been verified (Griffiths-Jones et al. 2006; Saini et al. 2007). Genomic analysis of regions around intergenic microRNAs identified putative transcriptional start sites and poly (A) tails (Saini et al. 2007). It is still not clear why the nascent pri-microRNA transcript can be several kilobytes in length considering only 80-100 nucleotides are processed further. It suggests there are unknown regulatory elements within the pri-microRNA transcript that influence its transcription. There are numerous predicted regulatory elements located upstream of microRNA genes, which may participate in regulating microRNA transcription but many remain to be characterised (Lee et al. 2007). Putative transcriptional start sites and transcription factor binding sites have been identified for primary microRNAs but many of these remain to be verified (Saini et al. 2007). Transcriptional factors including c-Myb, NF-Y, Sp-1, MTF-1, and AP-2α are potential regulators of microRNA transcription. Interestingly these have been previously associated with diseases such as cancer (Lee et al. 2007). In addition, transcription factors activated by extracellular factors such as hormones may in turn activate primary microRNA transcription. For example, let-7 promoters appear to be activated by steroids via action on the nuclear receptor DAF12, resulting in down-regulation of let-7 targets (Bethke et al. 2009).

2.2.2 DROSHA and microRNA processing

Two years following the initial microRNA sequencing studies, several studies reported an RNase III enzyme was essential for initiation of microRNA processing (Han et al. 2004; Lee et al. 2003; Gregory et al. 2004; Denli et al. 2004). The RNase III enzyme DROSHA initiates microRNA processing in the nucleus (Lee et al. 2003).

DROSHA is the catalytic subunit of a large microprocessor complex (Figure 2-1), which cleaves pri-microRNAs leaving ~70 nt hairpin pre-microRNAs (Gregory et al. 2004). In-vitro microRNA processing reactions demonstrate immunoprecipitated DROSHA can cleave pri-microRNA to pre-microRNA (Lee et al. 2003). Furthermore, when DROSHA is blocked using RNAi, both pre- and mature microRNAs decrease but pri-microRNAs increase (Lee et al. 2003). RNAi against DROSHA resulted in down-regulation of six microRNAs profiled; miR-23a, miR-27b, let-7a-1, miR-16, miR-20 and miR-21 (Lee et al. 2003), thus establishing DROSHA as a key regulator of microRNA maturation. However, it is unknown whether DROSHA is critical for processing of all human microRNAs. In *Drosophila* S2 cells depletion of DROSHA

mRNA using dsRNA revealed 137 DROSHA regulated RNAs, although surprisingly only 11 pri-microRNAs (Kadener et al. 2009).

The RNA binding protein DGCR8 is another important component of the microprocessor complex (Figure 2-1), which is required for microRNA biogenesis (Gregory et al. 2004) and also helps stabilize the DROSHA protein (Han et al. 2009). DGCR8 recognizes pri-microRNA and directs DROSHA to the specific position for cleavage to pre-microRNA (Han et al. 2006). DEAD-box RNA helicase subunits are also part of the microprocessor complex (Gregory et al. 2004) and knockout of the DEAD-box RNA helicase subunits, p68 and p72 caused early lethality in mice (Fukuda et al. 2007). Interestingly, in p68 (-/-) and p72 (-/-) embryos, only a subset of microRNAs were affected, which suggests DEAD-box RNA helicase subunits may be involved in the recognition of specific pri-microRNAs for processing by DROSHA (Fukuda et al. 2007).

2.2.3 Nuclear export of pre-microRNAs

Following processing by DROSHA, pre-microRNAs are rapidly exported from the nucleus to the cytoplasm (Figure 2-1) via a GTP dependent nuclear transport protein (Bohnsack et al. 2004; Lund et al. 2004). Inhibition of nuclear transport proteins revealed EXPORTIN5 was responsible for the majority of microRNA export (Lund et al. 2004). Despite the prevailing view that microRNAs are actively exported from the nucleus, at least one study has demonstrated mature microRNA can be predominantly localised in the nucleus (Hwang et al. 2007). It is currently not clear how many microRNAs are predominantly localised in the nucleus or what their function is. In the case of miR-29, this has been shown to function in the cytoplasm leading to suppression of mRNA targets associated with myocardial infarction (van Rooij et al. 2008) and also linked to insulin resistance in adipocytes (He et al. 2007). The original experiments demonstrating export of microRNA via EXPORTIN5 reported seven individual microRNAs required this protein to accumulate in the cytoplasm (Lund et al. 2004). It has since been assumed EXPORTIN5 is responsible for all microRNA export from the nucleus as no selective regulation of microRNA export has been reported.

2.2.4 Pre-microRNA processing by DICER

Pre-microRNAs in the cytoplasm are processed by DICER (Figure 2-1) to produce functional mature microRNAs (Kim et al. 2009). This was demonstrated by *in-vitro*

experiments involving incubation of 70 nt pre-microRNAs with immunoprecipitated DICER. The result of these *in-vitro* experiments was that 22 nt mature microRNAs accumulated (Ketting et al. 2001). In addition, introduction of mutations in the DICER gene (DCR-1) blocked cleavage of dsRNA (Knight & Bass, 2001). Furthermore, other studies have clearly shown DICER is essential for global microRNA processing. For example, knockout DICER mutants are generally embryonic lethal, or result in severe phenotype abnormalities (Bernstein et al. 2003), and in ovarian cancer DICER expression levels predict survival (Merritt et al. 2008). However, changes in microRNA biogenesis protein expression are not always accompanied by increased mature microRNA expression, which suggests there must be other regulatory steps involved in determining mature microRNA expression.

2.2.5 RNA induced silencing complex (RISC)

The RISC consists of three main proteins, DICER, TRBP and ARGONAUTE (Kim et al. 2009) and their interaction is shown in Figure 2-1. TRBP is a double stranded RNA binding protein, which binds to the microRNA duplex following cleavage of the pre-microRNA by DICER (Chendrimada et al. 2005). The microRNA duplex unwinds and the guide strand is incorporated into the RISC while the passenger strand is rapidly degraded (Hutvágner & Zamore, 2002). The ARGONAUTE proteins are critical players in the formation and function of the RISC complex (Su et al. 2009). Most endogenous microRNAs are reportedly tightly bound to RISC complexes and few remain unbound in the cytoplasm (Tang et al. 2008).

2.2.6 ARGONAUTE proteins

Immunoprecipitation of AGO2 protein followed by microarray analysis revealed hundreds of microRNAs and mRNAs are bound specifically to AGO2 proteins (Rehwinkel et al. 2006). Knockdown of AGO2 protein is reported to up-regulate several hundred mRNAs with significant enrichment of mRNAs containing putative microRNA binding sites (Rehwinkel et al. 2006). Transfection of AGO proteins in human HEK 293 cells followed by co-immunoprecipitation revealed known proteins such as DICER and TRBP bound to AGO, but also proteins containing DEAD box motifs, heterogenous nuclear ribonucleoprotein particles, messenger RNA binding proteins and proteins involved in RNA metabolism (Höck et al. 2007). The role of many of these AGO-associated proteins in RISC silencing has not yet been established. Knockdown of RBM4 protein revealed it was necessary for microRNA-guided gene

regulation (Höck et al. 2007). Recently, IMP8 was identified as another protein required for microRNA regulation. IMP8 interacts with AGO2 proteins and knockdown of IMP8 affects AGO2 associated mRNAs (Weinmann et al. 2009).

2.2.7 P-bodies

Repressed mRNA and microRNA can collect in cytoplasmic P-bodies (Figure 2-1), which contain enzymes involved in mRNA degradation, such as deadenylases and decapping enzymes (Liu et al. 2005; Jackson & Standart, 2007). These enzymes are recruited to P-bodies by GW182. ARGONAUTE proteins contain multiple GW182 binding sites (Takimoto et al. 2009) and AGO1 can interact with GW182 thus bringing microRNA targets into close proximity with mRNA degradation enzymes (Eulalio et al. 2008). Repression of microRNA targets in *C.elegans* is reportedly dependent on GW182 proteins. Furthermore, in Drosphila knockdown of GW182 is reported to alleviate microRNA mediated translational repression (Behm-Ansmant et al. 2006). P-bodies may also act as temporary storage depots for untranslated mRNA, which would explain translational repression in the absence of mRNA cleavage. However, it is not currently clear whether untranslated mRNA localised in P-bodies can be later released and translated.

2.3. Blockage in microRNA processing

A large-scale profiling study of different tumour and tissue cell lines revealed widely differing microRNA processing (Lee et al. 2008). Some microRNAs were processed efficiently, demonstrated by a strong correlation between pre- and mature microRNA abundance (Lee et al. 2008). In contrast there was discordant expression of pre- and mature forms for other microRNAs. For example, there appeared to be an accumulation of pre-miR-31 in cancer cells, as mature miR-31 abundance was much lower (Lee et al. 2008), which suggests microRNA processing may be blocked in cancer cells at least for some microRNAs. A down-regulation of DROSHA and DICER could help explain evidence of decreased microRNA processing. However, any reduction in DROSHA or DICER activity would result in a whole-cell reduction in mature microRNA expression, which would be expected to lead to fundamental changes in cell phenotype.

2.3.1 RNA binding proteins

Studies published recently have revealed other factors can influence processing of individual microRNAs. For example, NF90 and NF45 proteins appear to function as negative regulators of microRNA processing. NF90 and NF45 are nuclear proteins, which bind pri-microRNAs and depletion of NF90 is reported to decrease pri-let-7 but increase mature let-7 expression, thus implicating NF90 as a negative regulator of microRNA processing (Sakamoto et al. 2009). Furthermore, recently BMP2 treatment was shown to stimulate pri-miR-206 expression but mature miR-206 was decreased (Sato et al. 2009), transfection of SMAD1 and SMAD4 both involved in BMP signaling also down-regulated miR-206 (Sato et al. 2009), thus suggesting SMAD proteins may modulate processing of specific microRNAs.

Several studies have shown RNA-binding proteins can be critical for microRNA processing (Kim et al. 2009). MicroRNAs can bind to hnRNP A1 prior to DROSHA processing and depletion of hnRNP A1 in HeLa cells appears to decrease pre-miR-18a abundance (Guil & Cáceres, 2007), indicating that pri-microRNA processing may be dependent on hnRNP A1. Intriguingly, it has been reported that 14% of all pri-microRNAs have highly conserved loops and it is suggested these may act as landing pads for RNA-binding proteins (Michlewski et al. 2008), but this remains to be experimentally verified. Another example of an RNA binding protein inhibiting microRNA processing is the inhibition of pri-let-7 by LIN28 (Viswanathan et al. 2008). In embryonic stem cells, LIN28 binds to a conserved region in the microRNA stem loop encoding let-7, which inhibits the activity of DROSHA and DICER, preventing embryonic stem cell differentiation (Viswanathan et al. 2008). RNA binding proteins have also been reported to bind to microRNA target sites. Dead end 1 (Dnd1) protein has been shown to bind to mRNA 3'UTRs and block microRNA function in human and zebrafish germ cells (Kedde et al. 2007). Furthermore, SMAD proteins could be involved in microRNA maturation, as both TGF-beta and BMP signaling which are responsive to SMAD, increased expression of mature miR-21 (Davis et al. 2008). MicroRNA processing by DROSHA was reportedly increased via recruitment of TGF-beta specific SMAD signal transducer proteins to pri-mir-21 (Davis et al. 2008). Taken together these studies suggest that RNA binding proteins may provide an additional layer of regulation in the microRNA processing pathway (Kim et al. 2009). However, most of the studies showing RNA-binding proteins influence microRNA processing are

based on *in-vitro* cell models, it remains to be seen if RNA-binding proteins are important for microRNA processing in humans *in-vivo*.

2.4. Mechanism of microRNA action

It has been shown that microRNAs can suppress protein levels through several mechanisms. MicroRNAs can bind to mRNA and cause cleavage, destabilisation, degradation of target mRNA or act via translation repression (Jackson & Standart, 2007).

2.4.1 MicroRNAs can act via target mRNA cleavage

MicroRNAs cause mRNA cleavage and degradation of target mRNAs based on several observations. In human cell extracts the RISC appears to be capable of multiple rounds of RNA cleavage (Hutvágner & Zamore, 2002). Depletion of microRNA processing proteins in human HEK293 cells increases mRNA abundance and many of the up-regulated mRNAs were reportedly enriched in sequences complementary to microRNA seed regions (Schmitter et al. 2006). Overexpression of specific microRNAs in HeLa cells reveals hundreds of transcripts are modulated, but the transcripts that were down-regulated were more likely to contain conserved microRNA binding sites (Lim et al. 2005). However, overexpression of microRNAs is likely to have many indirect effects (Bartel, 2009). For example, 30% of microRNAs are predicted to target transcription factors, which would indirectly affect mRNA abundance (John et al. 2004). Taken together these studies indicate microRNAs can cause cleavage or degradation of many mRNAs.

The first direct evidence that microRNAs could function by mRNA cleavage was from RNA fragments detected in mouse embryos that corresponded to HOXB8 (Yekta et al. 2004). The HOX cluster also encodes miR-196 and HOXB8, HOXC8 and HOXD8 all contain evolutionary conserved miR-196 sites. Cell culture experiments demonstrated miR-196 knockdown led to a decrease in HOXB8 cleavage fragments (Yekta et al. 2004), thus indicating miR-196 can promote cleavage of HOXB8 and hence post-transcriptionally suppress HOXB8 protein. Furthermore, another example of microRNA mediated mRNA degradation has been reported in zebrafish, miR-430 appears to be responsible for clearance of mRNAs during early embryogenesis (Giraldez et al. 2006). In zebrafish, maternal mRNAs are targeted by miR-430 resulting in deadenylation and degradation (Giraldez et al. 2006). Deadenylation appears to be a

widespread mechanism for microRNA mediated target degradation. A recent study compared mRNA profiles from AGO1, CAF1 or NOT1 depleted cells. CAF1 and NOT1 are both proteins required for deadenylation of mRNAs (Eulalio et al. 2009). Interestingly, 60% of AGO1 targets were also regulated by CAF1 or NOT1, which suggests that many microRNAs may act via deadenylation (Eulalio et al. 2009).

2.4.2 MicroRNAs can act via translational repression

In mammalian cells mRNA cleavage requires perfect complementarity between the microRNA seed and the mRNA target 3'UTR (Bartel, 2004; Bartel, 2009). However, most microRNA binding sites in humans have imperfect complementarity. Therefore, in mammalian cells translational repression is most likely to be the main mechanism for microRNA mediated silencing. Translation consists of three major steps - initiation, elongation and termination - and is the process by which mRNAs are translated into proteins. Translational repression of microRNA targets appears to involve inhibition of translation initiation, as *in-vitro* experiments show let-7 microRNA is able to repress translation initiation within 15 min and without mRNA destabilization, by targeting the cap-binding complex eIF4F (Mathonnet et al. 2007). Action of microRNAs by translational repression would therefore be undetectable at mRNA level and could only be detected at protein level using, for example, Western blotting.

2.4.3 MicroRNAs can be translational activators

The prevailing view is that microRNAs act as translational repressors. However, some data suggest microRNAs can be involved in translational activation. Interaction of an AGO2/FXR1 complex with mRNA 3'UTRs leads to up-regulation rather than down-regulation of translation (Vasudevan et al. 2007), in addition microRNA action appears to be dependent on cell cycle. For example in proliferating mammalian cells, upon cell cycle arrest microRNAs which normally repress translation may induce translational up-regulation of target mRNAs (Vasudevan et al. 2008). Currently, there are no other reports of microRNAs inducing translational up-regulation of target mRNAs.

2.5. Methods to quantify microRNA expression

The huge interest in microRNA regulation of gene expression in diseases such as cancer has led to rapid developments in technology to measure microRNA expression (Cissell & Deo, 2009). The earliest studies on microRNA function in *C.elegans* and *Drosophila* used solely Northern blots (Ambros et al. 2003; Lagos-Quintana et al. 2001). Northern

blots remain the gold standard despite being impractical for use in human samples due to the large RNA input required (Válóczi et al. 2004). Now real-time quantitative PCR (RT-qPCR) is commonly used and a range of custom and commercial microRNA arrays has been developed (Cissell & Deo, 2009; Li & Ruan, 2009). However, Northern blots do have advantages for detection of different microRNA precursors (Kim, 2004). Indeed it was the appearance of an additional signal at ~70 nt that led to speculation that mature microRNA were not transcribed as 22 nt sequences, but were processed from longer transcripts (Lagos-Quintana et al. 2001). Northern blot may be limited when sensitivity is required to detect small differences between human RNA samples (Cissell & Deo, 2009). Furthermore, Northern blot does not allow high-throughput measurement of many microRNAs so is not feasible for global tissue profiling (Cissell & Deo, 2009).

2.5.1 MicroRNA detection using real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) does provide the possibility to measure microRNA expression with higher sensitivity and higher-throughput than Northern blot (Cissell & Deo, 2009). Due to the small length of microRNAs and to increase specificity a two-step RT-qPCR protocol is widely used (Chen et al. 2005), which involves an RT step that instead of using random hexamer primers uses stem-loop primers specific to mature microRNA sequences (Chen et al. 2005). The use of microRNA specific stem-loop primers facilitates higher amplification efficiency during the second RT-qPCR step. There are several advantages of using RT-qPCR for detection of microRNAs. Firstly, only a small RNA input is required: as low as 2 ng/µl compared to 10 µg needed for Northern blots. Secondly, RT-qPCR is very sensitive to differences in microRNA abundance over a wide dynamic range (Chen et al. 2005). Thirdly, RT-qPCR is very specific and should be able to differentiate between mature microRNAs from the same family differing by a single nucleotide (Chen et al. 2005; Cissell & Deo, 2009). Typically, an endogenous small RNA is used to control variations in cDNA synthesis during the RT step, so it is important to determine whether the endogenous small RNA used is stably expressed across all experimental conditions (Davoren et al. 2008). RT-qPCR provides a medium-throughput method to determine mature microRNA abundance and generally shows good agreement with Northern blot (Chen et al. 2005).

2.5.2 MicroRNA detection using microarrays

Rapid developments have been made in designing microarray chips to detect global changes in microRNA profiles (Kong et al. 2009; Liu et al. 2008; Cissell & Deo, 2009). These chips work on a similar basis as oligonucleotide arrays, except they contain probes designed to hybridise to small RNAs. There are an ever-growing number of commercial and custom microRNA arrays that have been used in different studies, which makes comparing studies difficult (Sato et al. 2009). Usually studies involving microRNA arrays attempt to validate findings using RT-qPCR or Northern blot for microRNAs of interest that are differentially expressed (Sato et al. 2009). Recently, a study compared agreement of microRNA array results across five platforms and concluded there was a lack of concordance between different platforms (Sato et al. 2009). It is largely unknown what effect different RNA sample preparation methods may have on microRNA expression profiles (Wang et al. 2008), or the effect of different normalization techniques on the subsequent determination of differentially expressed microRNAs (Sato et al. 2009), or how sensitive microRNA arrays are to small differences in microRNA abundance (Sato et al. 2009). Recently, the use of additional spike-in controls in microRNA chips was suggested to provide a measure of chip-to-chip variation (Sarkar et al. 2008). Unfortunately, microRNA arrays are still a relatively expensive technology, which precludes routine use in laboratories or for clinical diagnostics (Cissell & Deo, 2009).

2.6. MicroRNA function and target prediction

Non-coding microRNAs consist of only 1-2% of the human genome (Lim et al. 2003). However, small non-coding RNA molecules may regulate over one third of protein coding transcripts. Identification of mRNAs with conserved binding sites that are complementary to microRNA seed sequences revealed over 5000 human genes may potentially be regulated by microRNAs (Lewis et al. 2005). Recently, the regulatory impact of microRNAs on the human genome was suggested to be even more widespread. MicroRNA target predictions using a more recent version of the human genome with better annotation suggests over 60% of protein coding genes have been under evolutionary pressure to maintain microRNA target sites (Friedman et al. 2009). Over 45,000 conserved microRNA target sites have now been identified in human 3'UTRs (Friedman et al. 2009).

2.6.1 Tissue-specific microRNAs

Analysis of mRNA target expression across tissues revealed that when a microRNA is highly expressed in a tissue, corresponding targets are expressed at a significantly lower level compared to other tissues (Sood et al. 2006). In contrast, when a microRNA is not expressed highly, target mRNAs are significantly more abundant (Sood et al. 2006). In skeletal muscle several microRNAs are highly expressed, including miR-1, miR-206 and miR-133. These have previously been shown to regulate skeletal muscle proliferation and differentiation (Chen et al. 2006). Tissue specific microRNAs appear to target non-tissue related genes for post-transcriptional repression during development. However, the function of tissue-specific microRNAs in adult skeletal muscle is not fully understood. It appears muscle-specific microRNAs are modulated during hypertrophy (McCarthy & Esser, 2007) and atrophy (McCarthy et al. 2007). The functions of muscle-specific microRNAs will be discussed further in section 2.11.

2.6.2 Prediction of microRNA targets

There are many microRNA target prediction algorithms, which predict microRNA targets across different species. The most established and widely used are TargetScan, PicTar and Miranda (Lall et al. 2006; John et al. 2004; Lewis et al. 2003). Each uses a different set of rules to identify and evaluate the efficacy of a microRNA target using a unique scoring system (Sethupathy et al. 2006).

Evaluating the utility of each prediction algorithm is difficult as there is no comprehensive set of experimentally validated targets (Bartel, 2009). Each algorithm must first identify potential binding sites according to specific base-pair rules. TargetScan requires perfect complementarity between 3'UTRs and microRNA seed sequences (Lewis et al. 2003), whereas imperfect seed matches are included in PicTar (Lall et al. 2006). The microRNA seed is defined at nucleotide 2-7 of the mature microRNA and is the main binding point of targets (Bartel, 2004; Lewis et al. 2003). It has been established that perfect complementarity between microRNA seed sequence and 3'UTR increases the likelihood of target repression (Bartel, 2004), but imperfect seed matches can also lead to target repression. Interestingly, the importance of the microRNA seed sequence as the main site for microRNA:mRNA target interactions was challenged recently. Bantam microRNA was reported to bind the 3'UTR of hid at nucleotide 3-9 in Drosphila S2 cells (Nahvi et al. 2009), which suggests that current target predictions may be missing valid microRNA targets.

Cross-species conservation is another important criterion for prediction of microRNA binding sites (Sethupathy et al. 2006). Evidence of cross-species conservation suggests there may be evolutionary pressure to retain functional microRNA binding sites (Bartel, 2004). TargetScan and PicTar both require conservation between at least five species (Lewis et al. 2003; Lall et al. 2006) to help limit false-positive predictions. TarBase contains evidence of experimentally validated microRNA targets (Sethupathy et al. 2006; Papadopoulos et al. 2009). Interestingly some are non-conserved microRNA targets, therefore would not have been predicted using PicTar or TargetScan, which suggest non-conserved microRNA binding sites can also be functional. Recently, TargetScan was updated and now provides predictions of non-conserved target sites (Friedman et al. 2009). However, it is not known how important non-conserved microRNA targets are to post-transcriptional regulation of human protein levels. The consensus among microRNA researchers is that experimental validation of predicted microRNA targets remains necessary to confirm the functional relationship between a microRNA and its target mRNA (Bartel, 2009; Ambros, 2004; Bushati & Cohen, 2007).

2.6.3 Experimental validation of microRNA targets

There is still no clear consensus on criteria to validate microRNA targets (Kuhn et al. 2008). A range of molecular methods have been employed to provide evidence of individual microRNA target interactions or less frequently genome-wide microRNA target interactions (Bartel, 2009). Many of the current microRNA target prediction algorithms were based on studies of mRNA and microRNA profiling. For example, Lim et al. (2003) presents correlations between microRNA and mRNA expression in response to microRNA knockdown as evidence of microRNA mediated target suppression. However, studying microRNA action on mRNA expression assumes microRNAs act primarily through mRNA cleavage (Bartel, 2009), but it is now known that microRNAs can target mRNAs for translation repression and so mRNA transcripts may remain unchanged. Translation repression is still suggested to be the main mechanism of microRNA action in mammalian cells (Kim et al. 2009).

Several experiments can be conducted to show a functional relationship between a microRNA and target mRNAs. It is important to confirm binding of microRNA to mRNA (Kuhn et al. 2008). This can be done by dual transfection of microRNA knockdown oligonucleotides and 3'UTR vectors containing the firefly luciferase gene,

followed by comparison of luciferase activity between cells transfected with 3'UTR vectors or control vectors without putative microRNA binding sites. Next it is important to establish that changing microRNA availability affects protein targets, by either microRNA overexpression in cells using synthetic microRNAs, or microRNA knockdown using anti-microRNA oligonucleotides and measuring changes in target protein level. Unfortunately, this approach to experimental validation of microRNA targets is not useful to assessing multiple targets, hence why so few predicted microRNA targets have been experimentally validated.

There are new approaches to identify many functional microRNA targets simultaneously based on co-immunoprecipitation of RISC proteins such as AGO2 or GW182 (Easow, Teleman, & Cohen, 2007; Zhang et al. 2007). Global protein profiling has been attempted in two recent studies (Baek et al. 2008; Selbach et al. 2008). In the first study, miR-223 was deleted in mice and the effect on protein measured using quantitative mass spectrometry in cultured neutrophils (Baek et al. 2008). Deletion of miR-223 in mice revealed hundreds of genes were directly repressed by miR-223, although some gene targets were repressed with no change in mRNA abundance. The most significant finding was that microRNAs appear to act as cellular rheostats and fine-tune protein levels (Baek et al. 2008). In the second study stable isotope labeling in cell culture was used after microRNA transfection or endogenous microRNA knockdown (Selbach et al. 2008). It was concluded that microRNAs function by tuning protein synthesis of thousands of genes (Selbach et al. 2008). Although these studies provide an opportunity to uncover new rules governing microRNA target interaction, there are still unanswered questions about the importance of tissue specific microRNA expression. For example, miR-133 is known to regulate SRF in cardiac muscle (Zhao et al. 2005), but it is not known if SRF is also regulated by miR-133 in skeletal muscle, adipose, brain or nervous tissue. Therefore, it is important that each microRNA:target interaction is validated in different tissues. Evidence of a microRNA:mRNA interaction in the heart may not apply in other tissues, such as adipose, liver or skeletal muscle tissue.

2.7. Skeletal muscle microRNAs

Human diseases characterised by muscle deterioration or dysfunction have only recently been studied at the post-transcriptional level (Eisenberg et al. 2007; Fredriksson et al. 2008). Studies within the past five years have revealed microRNAs are important

regulators of muscle development (Nguyen & Frasch, 2006) and there is now evidence to suggest microRNAs may be involved in many biological processes influencing muscle growth (van Rooij et al. 2008), muscle wasting and remodeling (Yang & Wu, 2007). However, most of our current knowledge of microRNA function is from model organisms or cell lines. Most studies tend to take a single gene/microRNA approach, which fails to appreciate that microRNAs may regulate >200 transcripts and that more than one microRNA determines cell phenotype *in-vivo* (Bartel, 2009). Studies on microRNA function in human skeletal muscle are surprisingly lacking despite their known influence in regulating gene expression.

2.7.1 MicroRNAs are involved in skeletal muscle development

Skeletal muscle development models have been used to characterise the role of microRNAs in proliferation and differentiation. In skeletal and cardiac muscle, miR-1, miR-133 and miR-206 are all highly expressed. Chen et al. (2006) identified miR-133a was able to drive proliferation of murine skeletal muscle C2C12 cells, while miR-1 and miR-206 were able to accelerate differentiation.

MicroRNA-1 is highly conserved between nematodes, flies and vertebrates, where it is highly expressed in muscle tissue (Lagos-Quintana et al. 2001). Tissue specific expression of a microRNA is generally assumed to indicate it may be important for development or maintenance of tissue phenotype (Sood et al. 2006). Ectopic expression of miR-1 in HeLa cells revealed that most of the target genes that were down-regulated are usually expressed at low-levels in skeletal muscle (Lim et al. 2005), which suggests miR-1 may be important for suppressing non-muscle genes to maintain tissue identity. Loss of miR-1 would be expected to lead to impaired muscle function and indeed it has been reported that *Drosophila* miR-1 mutants have severely deformed musculature (Sokol & Ambros, 2005). In cardiac muscle, there is evidence that miR-1 may regulate proliferation and differentiation by targeting HAND2 (Mishima et al. 2007), as HAND2 is a transcription factor essential for proliferation of cardiac cells. In developing mouse hearts, ectopic expression of miR-1 led to down-regulation of HAND2 (Mishima et al. 2007). Knockdown of miR-1-2 was reported to have opposite effects, stimulating cardiogenesis (Zhao et al. 2007). However, analysis of different sections of developing mouse hearts revealed HAND2 was down-regulated in part of the right ventricle whereas miR-1 was highly expressed (Zhao et al. 2007). These findings highlight that even within a single tissue microRNA expression can vary. It is difficult to extrapolate

these findings to skeletal muscle, as HAND2 has not been shown to be an essential factor in myogenesis. It is likely the relationship between a microRNA and a target gene is context specific (Bartel, 2009). For example, microRNA target suppression in pancreatic cells may not occur in skeletal muscle even where both microRNA and target are expressed.

In *C elegans*, miR-1 has been shown to regulate the muscle transcription factor MEF2 at the neuromuscular junction (Simon et al. 2008). MEF2 increases during skeletal muscle differentiation and it has been demonstrated that MEF2 can activate transcription of the primary transcript encoding miR-1-2 and 133a-1 (Zhao et al. 2007; Liu et al. 2007). Pri-miR-1-2 and pri-miR-133a-1 increase significantly during muscle differentiation (Brzeszczynska et al. under revision; Rao et al. 2006). Therefore, miR-1 appears to be part of a more complex regulatory network, possibly acting as its own negative regulator by targeting MEF2. However, it is unlikely MEF2 is solely responsible for determining miR-1 transcription, as additional transcription factor binding sites have been identied on the primary transcripts encoding miR-1 (Rao et al. 2006). MicroRNA-1 appears to promote mesoderm formation from both mouse and human embryonic stem cells in unison with miR-133 under the regulation of serum response factor (Ivey et al. 2008). Interestingly, in later development during differentiation into cardiac muscle progenitors these microRNAs are reported to have opposing functions (Ivey et al. 2008).

Muscle microRNAs appear to respond to extra-cellular factors, for example oxidative stress reportedly increases miR-1 expression in cardiomyocytes, which corresponded to suppression of the miR-1 targets HSP60 and HSP70 promoting apoptosis (Xu et al. 2007). In contrast, miR-133 was reported to target CASP9 and protect cardiomocyctes from apoptosis (Xu et al. 2007). Interestingly, it has been suggested both miR-1 and miR-133 are transcribed in unison from neighbouring transcripts (Chen et al. 2006). Evidence of opposing effects on apoptosis raises the question whether miR-1 and miR-133 are indeed transcribed in unison but processed at different rates. A similar situation arises during skeletal muscle hypertrophy where mature miR-1 and miR-133 are differentially expressed despite originating from the same genomic loci (McCarthy & Esser, 2007) and recently differential expression of miR-133a and miR-1 has been reported after endurance training in rats (Safdar et al. 2009). It may be worthwhile examining the expression of primary microRNAs encoding miR-1 and miR-133 and

determining whether these microRNAs with apparently differing functions in skeletal muscle are indeed transcribed and processed together. Taken together these studies suggest microRNAs are involved in important biological functions in skeletal muscle including proliferation, differentiation and apoptosis. Furthermore, it suggests the highly expressed muscle specific miR-1, miR-133 and miR-206 may play a role in adult skeletal muscle possibly in maintaining muscle phenotype but also potentially under pathological conditions such as cancer and Type 2 diabetes. However, studies on the role of these microRNAs in human skeletal muscle are lacking.

2.7.2 MicroRNAs and skeletal muscle adaptation

In healthy skeletal muscle, remodeling occurs in response to exercise training. In human skeletal muscle over one thousand transcripts were reportedly changed with endurance training (Timmons et al. 2005). Intriguingly, a follow-up study found twenty-one microRNAs were differentially expressed in response to endurance training (Keller et al. 2007). The predicted targets of these microRNAs shared similar gene ontology groups to those of the ~1000 differentially expressed mRNAs (Keller et al. 2007). Gene-set enrichment analysis revealed up-regulation of the transcription factors RUNX1, PAX3 and SOX9 appeared to modulate the transcriptome response to training. Interestingly, these transcription factors are targets of five microRNAs (miR-101, -144, -1, -206 and -92) down-regulated in response to training (Keller et al. 2007). Based on our current understanding of microRNA function, Keller et al. suggest microRNAs may play an important role in muscle adaptation to training. However, recent findings from mice following endurance training found miR-1 was down-regulated but miR-133a was unchanged (Safdar et al. 2009). Nevertheless, these studies suggest microRNAs play a role in skeletal muscle remodelling in response to training, but more studies are needed to determine whether microRNAs are the cause or an effect of the remodelling process.

2.7.3 MicroRNAs and skeletal muscle hypertrophy

Given that microRNAs appear to be critical for skeletal muscle differentiation, it is possible microRNAs play a role in skeletal muscle hypertrophy. Signaling pathways associated with hypertrophy have already been identified (Bassel-Duby & Olson, 2006). For example, it is known that during recovery from resistance exercise, growth pathways are activated including mTOR and S6K (Atherton et al. 2005), activation of these pathways is associated with an elevation in protein synthesis. In rodent skeletal muscle, expression of mature miR-1 and miR-133a is reportedly down-regulated by

~50% following 7 days of functional overload (McCarthy & Esser, 2007). However, expression of the microRNA precursors encoding miR-1, miR-133, and miR-206 were all elevated, which suggests some changes in microRNA processing may be occuring. Counter-intuitively, DROSHA and EXPORTIN5 transcript levels were significantly increased (McCarthy & Esser, 2007), suggesting another factor must be responsible for altered microRNA processing. The change in miR-1 and miR-133a expression in response to skeletal muscle hypertrophy conversely raises the question whether microRNAs may also play a role during atrophy and skeletal muscle wasting.

2.7.4 MicroRNAs and skeletal muscle atrophy

Eisenberg et al. (2007) conducted microRNA profiling in skeletal muscle across a range of primary muscular disorders. Five microRNAs (miR-146b, miR-155, miR-214, miR-221 and miR-222) were reportedly dysregulated across ten muscular disorders (Eisenberg et al. 2007), which suggest microRNAs may play a role in their development. In Duchenne's Muscular Dystrophy the biological functions targeted by the down-regulated mRNAs were reportedly correlated with biological functions of the targets containing binding sites for the up-regulated microRNAs (Eisenberg et al. 2007). Over fifty microRNA-mRNA interactions were identified in muscular dystrophy, including proenkephalin-miR-29c, trophinin-miR-29c, RUNX1-miR-30a-5p and PDE4D-miR-199a. Unfortunately, these predicted microRNA:mRNA interactions have not been experimentally validated. Surprisingly, no significant interactions were found between microRNA expression and changes in genes coding proteins such as dystrophin, previously identified as a key player in muscular dystrophy (Eisenberg et al. 2007), which highlights the need to focus on changes in global gene expression rather than expression of single genes.

Muscle function is impaired in various chronic diseases characterized by muscle wasting and loss of glycaemic control including cancer and Type 2 diabetes. Despite the reported widespread influence of microRNAs on molecular, cellular and biological function, the role of microRNAs in skeletal muscle of cancer and Type 2 diabetes patients is unknown.

2.8. Cancer cachexia

Cachexia is a complex syndrome, which is characterised by progressive losses of both skeletal muscle and adipose tissue (Skipworth et al. 2007). Some degree of weight loss

is experienced by about 50% of all cancer patients and cachexia has been reported to be a significant contributing factor to mortality rates in cancer patients (DeWys, 1985). Recently, the SCRINO working group classified cancer patients with <10% weight loss as asymptomatic pre-cachexia and patients with >10% weight loss as symptomatic cachexia (Bozzetti & Mariani, 2008). In severe cases cachexia patients have been observed to lose 20-30% of initial body weight (Weber et al. 2009). Advanced cachexia is associated with anorexia, early satiety, severe weight loss, weakness, anemia and edema (Del Fabbro et al. 2006). It is common in patients with chronic or end-stage diseases such as sepsis, AIDS, congestive heart failure and cancer. It shares common features with diabetes, in that there is gross loss of metabolic control and therefore loss of proper insulin action (Rofe et al. 1994). In addition, cachexia has also been linked to a decreased response to chemotherapy treatment (DeWys, 1985).

Muscle wasting in cancer patients has major implications for quality of life and physical function (Stewart et al. 2006). Quality of life measures are reported to decline in association with weight loss. These changes also coincide with decreases in physical function (Dahele et al. 2007; Ryan et al. 2007; Fouladiun et al. 2007). Although, muscle wasting is the most identifiable characteristic of cachexia patients, many more factors may contribute to the manifestation of clinical symptoms of fatigue (Stewart et al. 2006). There is no clearly defined physiological profile of cancer cachexia patients and the pathogenesis of cancer cachexia is still not fully understood. Studies have reported many factors may be associated with cancer cachexia pathogenesis including alterations in protein, glucose and fat metabolism; circulating cytokines, tumour derived factors, and production of acute phase reactants (Skipworth et al. 2007; Melstrom et al. 2007; Baracos 2006; Laviano et al. 2005; Rubin, 2003).

2.8.1 Acute-phase response in cancer cachexia

An acute phase response is characteristic of many weight-losing cancer patients with tumour progression. The acute phase response represents a global response by the host-immune system to injury and involves the synthesis of acute phase proteins in hepatocytes (Stephens et al. 2008). C-reactive protein concentration in plasma is often used as an indicator of the degree of the acute phase response, for example Fearon et al. (1992) reported high C-reactive protein concentrations of 75 ml/l in a sample of colon-cancer patients. The degree of the acute phase response at cancer diagnosis has been reported to be a strong predictor of 5 year survival in colon cancer patients (Fearon,

1992). Furthermore, in pancreatic cancer patients the presence of an acute-phase protein response was found to be a significant independent predictor of survival duration (Falconer et al. 1995). Production of acute-phase reactants requires a supply of amino acids. If dietary intake is low as is often the case in cancer patients (Hutton et al. 2006) then amino acid requirements must be met by the breakdown of protein from other sources such as muscle. Thus it has been suggested the acute phase response may drive the breakdown of skeletal muscle proteins and lead to loss of muscle function (Reeds et al. 1994; Skipworth et al. 2007; Stephens et al. 2008). Nevertheless, the development of an acute phase response in cancer patients can not be the sole factor determining weight loss, as not all patients with metastatic disease, nor weight-losing cancer patients exhibit an acute phase response (Fearon, 1992).

2.8.2 Cytokines and cancer cachexia

Several cytokines have been implicated in the acute-phase response. Both *in-vitro* and *in-vivo* studies have shown IL-1 and TNFα can produce an acute phase response, for example infusion of TNFα in cancer patients is reported to elevate serum C-reactive protein concentrations (Selby et al. 1987). Yet in cancer cachexia patients circulating TNFα and IL-1 have been reported to be very low or undetectable (Fearon, 1992). In contrast IL-6 concentrations were significantly increased in cancer cachexia patients compared to controls. Unfortunately, there is a lack of consistency between studies on cytokine levels in cancer patients, thus cytokines may be of limited use as biomarkers of cachexia (Tan & Fearon, 2008).

Cancer patients with cachexia tend to have elevated levels of IL-6 in plasma (Fearon et al. 1991). Infusion of IL-6 into non-tumour bearing mice is reported to affect muscle protein levels. For example, myofibrillar protein was reportedly reduced by 17% over 14 days due to IL-6 infusion in non-tumour bearing mice (Haddad et al. 2005). In tumour-bearing mice circulating IL-6 has been observed to be increased (Lönnroth et al. 1990), but in another study no elevation of IL-6 levels was found in tumour-bearing mice compared to pair-fed controls (Mulligan et al. 1992). The inconsistency between studies using tumour-bearing mice may be related to the type of tumour used, as differential gene expression has been observed in different tumour models (Monitto et al. 2001). In humans, IL-6 levels reportedly do not differ between cachexic and non-cachexic patients diagnosed with non small cell lung cancer (Kayacan et al. 2006). However, a review of clinical studies on targeted anti-IL-6 monoclonal antibody

therapy in cancer patients revealed treatment was effective in decreasing CRP and was also associated with a decrease in the incidence of cachexia (Trikha et al. 2003). Taken together these studies suggest cytokines may play a role in cachexia pathogenesis, by activating an acute phase response or triggering protein breakdown in skeletal muscle.

2.8.3 Activation of the ubiquitin-proteasome pathway in cancer cachexia

In atrophy conditions the ubiquitin-proteasome pathway appears to be critical for breakdown of myofibrillar proteins. Indeed, in head trauma, sepsis and AIDS, gene expression of the ubiquitin-proteasome pathway is reportedly increased (Mansoor et al. 1996; Tiao et al. 1997; Llovera et al. 1998; Williams et al. 1999). The degradation of proteins via the ubiquitin-proteasome pathway involves conjugation of ubiquitin to individual proteins, which are then selectively degraded in the proteasome. Ubiquitin activates an E1 enzyme and activated ubiquitin binds to an E2 enzyme, which allows interaction with an E3 enzyme. The E3 enzyme binds to the protein substrate to be degraded and catalyzes the transfer of the activated ubiquitin from the E2 carrier (Kwak et al. 2004). Finally, ubiquitin tagged proteins are targeted to the 26S proteasome where degradation occurs.

The ATP-ubiquitin-proteasome pathway is reportedly activated in skeletal muscle of cachectic rats bearing a hepatoma (Baracos et al. 1995). A later study found the expression of genes in the ubiquitin-proteasome proteolytic pathway was increased in skeletal muscle from patients with cancer (Williams et al. 1999). The mRNA levels for the ubiquitin and 20S proteasome subunits were reported to be 2-4 times higher in cancer patients compared to controls, although it is not clear whether gene expression was associated with patient status as only 12 patients were sampled. Only three of these patients exhibited weight loss, suggesting ubiquitin genes may be up-regulated prior to protein breakdown resulting in loss of muscle mass. As with all studies measuring mRNA levels, it is not clear whether changes in expression are indicative of increased transcription or increased mRNA stability. In addition, changes in mRNA do not exclude the possibility of further post-transcriptional regulation of the ubiquitin-proteosome or other pathways in cancer cachexia.

In atrophying muscle, molecular signaling pathways controlling protein synthesis may be dysregulated, but there are only limited data on the activation of molecular growth pathways in human cancer cachexia. Schmitt et al. (2007) examined activation of the

Akt pathway in muscle and liver biopsies from patients with pancreatic carcinoma. In skeletal muscle, Akt on Ther308, mTOR and p70S6 kinase protein content was significant reduced (Schmitt et al. 2007), which suggests protein synthesis pathways may be down-regulated in cancer cachexia and therefore can not compensate for the activation of protein degradation via the ubiquitin proteasome pathway.

2.8.4 Breakdown of myofibrillar proteins in cancer cachexia

Myofibrillar protein forms a large proportion of skeletal muscle and consists of the contractile proteins myosin heavy chain, actin, troponin and tropomyosin (Bassel-Duby & Olson, 2006). In healthy skeletal muscle, expression of genes encoding myofibrillar proteins is essential to maintain muscle mass, loss of muscle mass could be explained by decreased expression of genes encoding myofibrillar proteins. For example, Acharyya et al. (2004) observed that MyHC was significantly down-regulated as a result of muscle wasting in mice implanted with C-26 tumours, but found no changes in expression of tropomyosin, troponin, actin, actinin and myosin light chain. Intriguingly, lower MyHC protein expression in C-26 tumour bearing mice was not associated with lower MyHC mRNA (Acharyya et al. 2004), which suggests MyHC could be regulated post-transcriptionally in cachexia.

2.8.5 Tumour-derived factor in cancer cachexia

Proteolysis inducing factor (PIF) is released from some tumours and was first isolated from urine of cancer patients with cachexia (Todorov, 1996). PIF is a sulphated glycoprotein, which has been implicated as an important mediator of protein catabolism in cancer cachexia. There are now *in-vitro* and *in-vivo* studies supporting the role of PIF in cachexia pathogenesis. Tisdale and colleagues have shown elevation of PIF appears to trigger an intracellular signaling cascade resulting in activation of the proteasome pathway (Todorov, 1996). In C2C12 myotubes PIF has been shown to increase protein degradation and decrease protein synthesis (Smith et al. 1999). *In-vivo* studies on rat gastrocnemius have also demonstrated that PIF increases protein degradation (Lorite et al. 1997).

Apoptosis activity is reportedly enhanced by PIF in-vitro. For example, treatment of C2C12 myotubes increases the activity of apoptotic initiators caspases-8 and -9 and apoptotic effector caspases -2, -3 and -6 (Smith & Tisdale, 2003). Furthermore,

following PIF treatment there was evidence of DNA fragmentation and free nucleasome formation indicating cell apoptosis (Smith & Tisdale, 2003).

More recent *in-vivo* studies on the role of PIF in cancer cachexia patients have produced mixed results (Cabal-Manzano et al. 2001; Williams et al. 2004; Wieland et al. 2007). A cross-sectional study in gastrointestinal patients observed PIF correlated with weight loss (Cabal-Manzano et al. 2001). A longitudinal study in cancer patients reported a positive relationship between urinary PIF pattern and persistent weight loss over time (Williams et al. 2004). However, another more recent study failed to find any association between cachexia severity and urinary PIF concentration in patients (Wieland et al. 2007). Therefore the evidence suggests PIF may be of limited clinical value despite reports of elevated PIF levels in cachexia patients.

2.8.6 Biomarkers for early detection of cachexia

Review of the cachexia literature to date reveals many potential biomarkers including tumour-derived factors such as PIF and cytokines involved in the acute phase response. However, although these are often observed to be elevated in cachexic patients in cross-sectional studies, consistent evidence from longitudinal studies directly linking changes in these markers to functional deterioration and weight loss in cancer patients is lacking (Lelli et al. 2003). For example, one cross-sectional study evaluated the potential of myoglobin in plasma to predict cancer-related muscle wasting. The study found plasma myoglobin was associated with aerobic capacity and muscle cross sectional area (Weber et al. 2007). Lower muscle cross sectional area and aerobic capacity were reported in cachexia patients, but muscle wasting was not associated with increases in plasma myoglobin (Weber et al. 2007). Another cross-sectional study attempted to identify a bio-humoral profile that can characterise cachexic and non-cachexic cancer patients, it was reported lung cancer patients developing cancer cachexia displayed significantly increased plasma TNFα and reactive oxygen species levels (Fortunati et al. 2007). However, a prospective study would be essential to confirm any proposed biomarker could predict weight loss. There still appears to be no reliable biomarker of cancer cachexia (Tan & Fearon, 2008), thus more research is warranted to identify biomarkers that can be used to reliabily detect cachexia in its early stages.

2.9. MicroRNAs implicated in cancer cachexia

It is clear microRNAs are important players in skeletal muscle differentiation and there are indications that microRNAs play a role in skeletal muscle remodelling, growth and atrophy (van Rooij et al. 2008; Chen et al. 2006; Chen et al. 2009; McCarthy & Esser, 2007; McCarthy et al. 2007). It is plausible that muscle microRNAs may play a role in cancer cachexia development or could be biomarkers of cancer cachexia progression. As yet there have been no studies on microRNA expression in skeletal muscle of cancer cachexia patients.

Based on microRNA-mRNA interactions reported in muscular dystrophies (Eisenberg et al. 2007), it is likely microRNA-mRNA interactions will also be found in cachexic muscle. Of particular interest are the microRNAs predicted to target genes coding proteins in the ubiquitin-proteasome pathway that appears to be activated in cachexia (Melstrom et al. 2007; Williams et al. 1999). In immune cells NF-κb activation induces miR-146 expression (Taganov et al. 2006), which is interesting as NF-κb is activated in cachexia at least in experimental models.

Other important players known to be involved in skeletal muscle atrophy including the E3 ligases MAFbx and MURF1 could also be regulated post-transcriptionally (Chen et al. 2007). MAFbx and MURF1 both contain multiple predicted microRNA target sites, however none of these have been experimentally validated.

2.10. Type 2 diabetes

Type 2 diabetes is characterised by peripheral insulin resistance, hyperglycaemia, hyperinsulinaemia and pancreatic β-cell dysfunction (Muoio & Newgard, 2008). Type 2 diabetes is distinct from Type 1 diabetes where pancreatic β-cell failure leads to uncontrolled hyperglycemia without external administration of insulin. Both genetic and lifestyle factors have been implicated in the development of Type 2 diabetes (Muoio & Newgard, 2008).

2.10.1 Type 2 diabetes susceptibility genes

Genome-wide association studies have identified several novel candidate genes with single nucleotide polymorphisms (SNPs), which increase Type 2 diabetes susceptibility (Zeggini et al. 2008). A large meta-analysis combining gene-association scans from over 10,000 individuals of European descent identified a further six previously

unknown loci encoding JAZF1, CDC123, TASPAN8, THADA, ADAMTS9 and NOTCH2 (Zeggini et al. 2008). However, most of the variance in Type 2 diabetes susceptibility remains to be explained. Later studies have found these candidate genes are also associated with increased susceptibility in other populations in Asia and Africa (Takeuchi et al. 2009). Most of the Type 2 diabetes susceptibility genes identified so far are involved in insulin secretion from pancreatic β-cells (Zeggini et al. 2008). Thus individuals with a family history of Type 2 diabetes have a higher susceptibility of progressing to Type 2 diabetes due to inherited pancreatic β-cell dysfunction.

It remains to be seen whether these Type 2 diabetes susceptibility genes are associated with the development of insulin resistance and impaired glucose tolerance, which also play an important role in Type 2 diabetes pathogenesis. A recent study found only IGF2BP2 and SLC30A8 were associated with impaired insulin sensitivity and glucose tolerance, but accounted for less than 10% of the variance in insulin sensitivity and glucose tolerance (Ruchat et al. 2008). Furthermore, a meta-analysis of Type 2 diabetes risk loci in a pre-diabetic cohort could not find any significant associations between Type 2 diabetes susceptibility genes and the pre-diabetic phenotype characterised by impaired insulin secretion and insulin resistance (Staiger et al. 2008). Together these studies suggest either more Type 2 diabetes susceptibility genes remain to be discovered, or that a large proportion of the pre-diabetic phenotype is determined by other factors.

2.10.2 Glucose homeostasis and insulin resistance in Type 2 diabetes

Normally glucose homeostasis is tightly regulated through production of insulin, which stimulates glucose uptake in peripheral tissues (Muoio & Newgard, 2008). Insulin is secreted from pancreatic β-cells in response to elevated blood glucose. In healthy skeletal muscle, binding of insulin to the insulin receptor results in the translocation of glucose transporters to the cell membrane and increased skeletal muscle glucose uptake (Muoio & Newgard, 2008). However, the development of insulin resistance leads to peripheral tissues being less responsive to insulin (Bouzakri et al. 2005). The pancreatic β-cells attempt to compensate for insulin resistance via increased insulin secretion, which can be a factor causing hyperinsulinaemia in Type 2 diabetes patients (Muoio & Newgard, 2008). Insulin resistance can be an early indicator of the development of Type 2 diabetes. However, not all insulin resistant individuals will develop hyperglycaemia and conversely patients may develop Type 2 diabetes in the absence of high insulin resistance due to hereditary pancreatic β-cell failure.

Insulin resistance is not only associated with Type 2 diabetes but also obesity (Kahn et al. 2006), physical inactivity (Hamburg et al. 2007), muscle-loss (Rofe et al. 1994), cancer and aging (Scheen, 2005). Obesity, ageing and physical inactivity have all been identified as risk factors increasing susceptibility to Type 2 diabetes. Therefore, it is important to control for these known risk factors when attempting to understand the underlying mechanisms contributing to insulin resistance and Type 2 diabetes. Current understanding of insulin resistance suggests there may be a breakdown in the intracellular signaling pathway activated by insulin (Fröjdö et al. 2009).

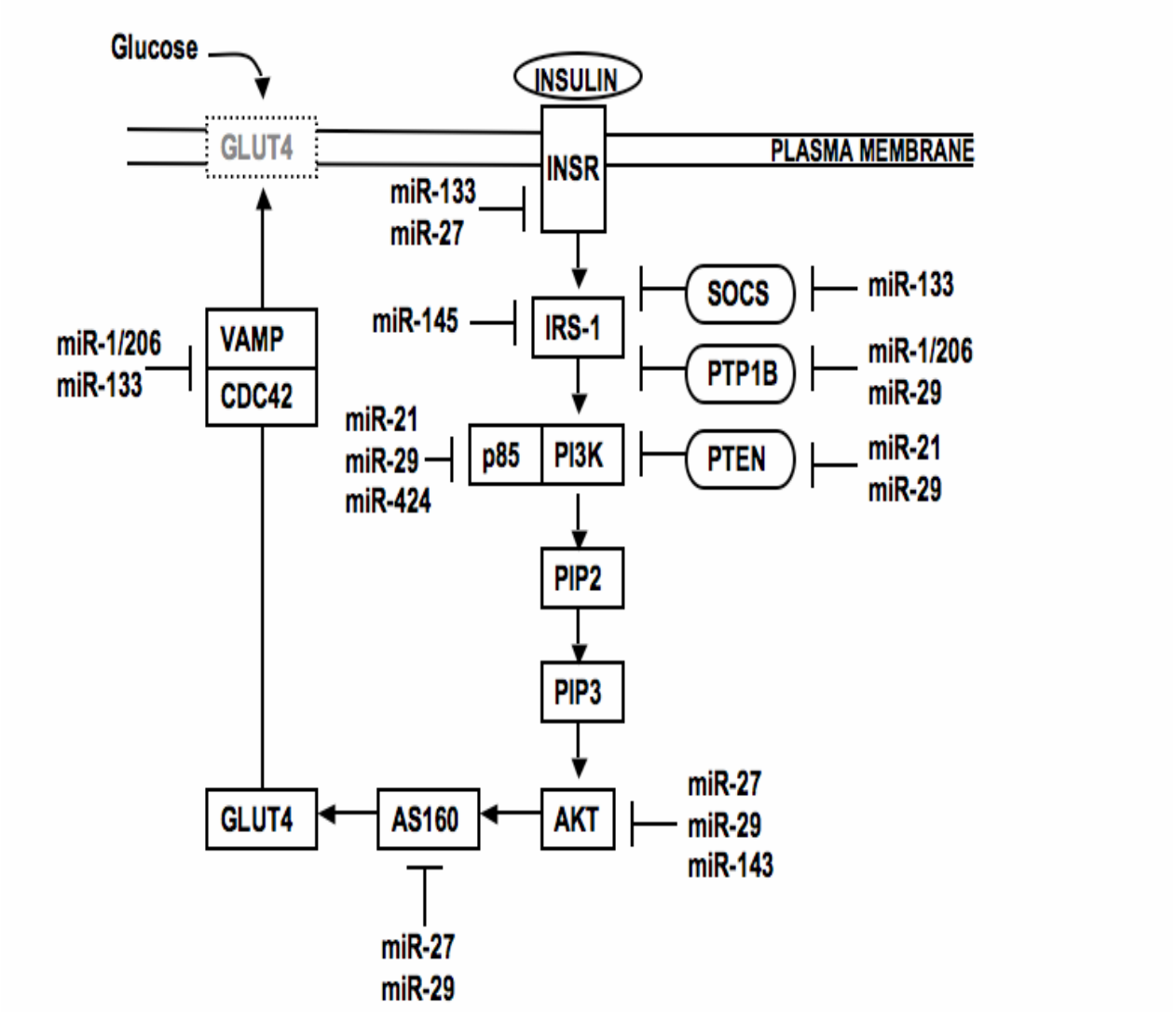


Figure 2-2. Insulin signaling pathway leading to skeletal muscle glucose uptake. Binding of insulin to the insulin receptor triggers a phosphorylation cascade. Activated of IRS1 triggers phosphorylation of PI3K. Conversion of PIP2 to PIP3 activates AKT, which in turn activates AS160 and results in translocation of GLUT4 to the plasma membrane. GLUT4 interacts with CDC42 and VAMP proteins may lead to increased glucose uptake. A selection of microRNAs predicted to target insulin signaling proteins are indicated.

2.10.3 Insulin signaling in skeletal muscle

Activation of the insulin signaling cascade in skeletal muscle is important to maintain normoglycaemia and may be impaired at multiple levels in Type 2 diabetes (Figure 2.2). In healthy skeletal muscle, insulin causes autophosphorylation of the insulin receptor (Burant et al. 1984). Docking of the insulin receptor substrate (IRS) proteins to the insulin receptor leads to phosphorylation of IRS proteins (Sun et al. 1991). Thereafter phosphorylated IRS proteins associate with phosphatidylinositol 3-kinase (PI3K) at the p85 regulatory sub-unit (Myers et al. 1992) and PI3K translocates to the plasma membrane. PI3K catalyses the conversion of phosphatidylinositol-4-5-biphosphate (PIP2) to phosphatidylinositol-3,4-5-triphosphate (PIP3), which results in binding, activation and co-localisation of phosphoinositide-dependent protein kinase-1 (PDK1), PDK2 and Akt (Stephens et al. 1998). Activated Akt results in phosphorylation of glycogen synthase kinase-3 (GSK3) and AS160, a RAB GTPase-activating protein (Kane et al. 2002). AS160 interacts with RAB10, a small GTPase, which facilitates the translocation of GLUT4 to the plasma membrane (Larance et al. 2005). GLUT4 vesicles are responsible for the majority of glucose uptake into human skeletal muscle. There are several negative regulators of insulin signaling, which may lead to decreased GLUT4 translocation and impaired insulin-stimulated muscle glucose uptake, thus potentially contributing to the development of insulin resistance and Type 2 diabetes.

2.10.4 Negative regulators of insulin signaling

Several points have been identified where insulin signaling may be impaired in Type 2 diabetes (Muoio & Newgard, 2008) and inflammatory cytokines have been shown to contribute to insulin resistance. Chronic systemic inflammation is a characteristic commonly associated with Type 2 diabetes and appears to contribute to the development of insulin resistance (Shoelson et al. 2006). For example, acute IL-6 administration in myotubes and mice has been reported to increase glucose uptake with activation of LKB1, PKB and AS160 (Nieto-Vazquez et al. 2008). However, chronic IL-6 administration has been reported to increase insulin resistance via decreased GLUT4 translocation and impaired IRS1 phosphorylation (Nieto-Vazquez et al. 2008). Furthermore, insulin resistance induced by chronic IL-6 exposure is associated with increased PTP1B activity (Nieto-Vazquez et al. 2008). PTP1B is a protein tyrosine phosphatase and can reverse autophosphorylation of the insulin receptor (Figure 2.2). It has been shown that myocytes deficient in PTP1B have increased insulin sensitivity

(Nieto-Vazquez et al. 2007), which suggests increases in PTP1B could result in insulin resistance.

Suppressor of cytokine signaling (SOCS) proteins can also act as negative regulators of insulin receptor tyrosine activity, which could lead to impaired insulin signaling (Krebs & Hilton, 2003). For example, insulin resistance induced by chronic IL-6 exposure is reported to activate JNK1/2 and increase SOC3 (Nieto-Vazquez et al. 2008), which may impair insulin induced IRS1 activation and hence contribute to insulin resistance.

PTEN is another negative regulator of insulin signaling, increases in PTEN triggers conversion of PIP3 to PIP2, which can attenuate insulin induced activation of PDK/AKT and reduce glucose uptake (Figure 2.2). PTEN could be involved in insulin resistance and Type 2 diabetes, as deletion of PTEN in muscle as been shown to protect mice from developing insulin resistance and Type 2 diabetes (Wijesekara et al. 2005). These studies highlight insulin signaling can be impaired at multiple levels leading to decreased insulin-stimulated glucose uptake.

2.11. MicroRNAs implicated in insulin resistance and Type 2 diabetes

Many insulin signaling genes have multiple microRNA binding sites in their 3'UTRs (Figure 2.2). Therefore microRNAs may regulate many stages in the insulin signaling pathway and play a role in the development of insulin resistance and Type 2 diabetes (Hennessy & O'Driscoll, 2008). Each microRNA is able to regulate multiple mRNAs, while each mRNA can be regulated by multiple microRNAs (Bartel, 2009). Despite the multiple microRNA binding sites in genes involved insulin-dependent glucose muscle uptake which is known to be impaired in Type 2 diabetes (Figure 2.2), to date there have been no studies on microRNA function in skeletal muscle of Type 2 diabetes patients.

2.11.1 MicroRNA-278 may contribute to an insulin resistant phenotype

It appears microRNAs have the potential to act as biomarkers of insulin resistance in muscle. *Drosophila* miR-278 mutants are reported to have an insulin resistant phenotype due to elevated insulin production, lower body mass and hyperglycaemia (Teleman et al. 2006). Although, miR-278 was reportedly expressed only in brain, gut, salivary gland and adipose tissue, but not skeletal muscle. Therefore, it appears miR-

278 may contribute to insulin resistance but not insulin resistance in skeletal muscle which is a characteristic of Type 2 diabetes.

2.11.2 MicroRNA-208 and microRNA-133 may target GLUT4

The glucose transporter GLUT4 is a predicted target of miR-208 (Figure 2.2). In response to miR-208 knockdown, if GLUT4 is a bon a fide target of miR-208 then GLUT4 protein should increase. However, in miR-208 knockout mice GLUT4 was found to be unchanged in cardiac muscle (van Rooij et al. 2007), although it is not clear whether miR-208 is expressed in skeletal muscle. It is known miR-133 is highly expressed in skeletal muscle and overexpression of miR-133 is reported to decrease the protein level of transcription factor KLF-15 and its downstream target GLUT4 (Takahiro, 2009). Conversely, silencing of miR-133 is reported to increase GLUT4 and increase cellular ATP (Takahiro, 2009). These studies suggest silencing of miR-133 or miR-208 could increase muscle glucose uptake and hence may be involved in the development of Type 2 diabetes. However, it would be important to establish miR-208 or miR-133 is altered in the skeletal muscle of Type 2 diabetes patients.

2.11.3 MicroRNA-143 targets diabetes associated genes

Diabetes-associated genes are reported to be inhibited by miR-143, which is expressed in both adipose and muscle tissue. For example, GLUT4 and peroxisome proliferator-activated receptor (PPAR-2) are reportedly inhibited by knockdown of miR-143 using 2'-O-methoxy anti-sense RNA oligonucleotides (Esau et al. 2004). In addition, mitogen-activated protein kinase ERK5, which can affect both PPAR-2 and GLUT4, was found to be a predicted target of miR-143 (Esau et al. 2004). It remains to be seen whether miR-143 acts on ERK5 in skeletal muscle and whether it is altered in Type 2 diabetes patients. ERK may be targeted by multiple microRNAs in Type 2 diabetes, as ERK activity appears to be modulated by miR-21 (Thum et al. 2008).

2.11.4 MicroRNA-21 targets insulin signaling regulators

MicroRNA-21 has been shown to inhibit ERK activity via suppression of sprouty homologue 1 (SPRY1) in heart fibroblasts (Thum et al. 2008). From a therapeutic perspective, administration of a miR-21 antagomir in mice with cardiac disease reportedly reduces ERK-MAP kinase activity and inhibits fibrosis (Thum et al. 2008). Furthermore, miR-21 is known to target PTEN (Figure 2.2), which regulates phosphoinositide 3-kinase signaling (Meng et al. 2007). Knockdown of miR-21 in

hepatocellular cancer cells increased PTEN expression confirming it as a target for repression by miR-21 (Meng et al. 2007). Fatty acids can down-regulate PTEN in hepatic tissue via activation of mTOR and NF-κB complex (Vinciguerra et al. 2008), interestingly activation of mTOR and NF-κB increases miR-21 promoter activity. In human liver biopsies from obese patients miR-21 was up-regulated (Vinciguerra et al. 2008), which points to miR-21 as a potential candidate for regulation in Type 2 diabetes. The responsiveness of miR-21 expression to extra-cellular events such as elevated free-fatty acids is particularly interesting as lipid infusion is associated with the development of acute insulin resistance. Furthermore lipid infusion has been reported to decrease IκB a known inhibitor of NFκB. Taken together these studies suggest elevated free-fatty acids in Type 2 diabetes may activate miR-21 transcription via NF-κB and increased miR-21 could suppress PTEN a negative regulator of insulin signaling. However, miR-21 is reportedly post-transcriptionally regulated by SMAD proteins, which can block maturation of pri-miR-21 (Davis et al. 2008). Therefore, it is difficult to make predictions about the role of miR-21 in Type 2 diabetes without further *in-vivo* evidence from Type 2 diabetes patients.

2.11.5 MicroRNA-145 targets insulin signaling protein IRS1

The insulin signaling IRS proteins are also microRNA targets (Figure 2.2) and IRS deficient mice reportedly develop insulin resistance (Kadowaki, 2000). IRS1 protein is decreased in response to overexpression of miR-145 in colon cancer cells, with IRS1 mRNA unchanged, and the direct interaction between miR-145 and IRS1 was confirmed using a luciferase reporter assay (Shi et al. 2007). However, it is not known whether miR-145 is a regulator of IRS1 in skeletal muscle and whether knockdown of miR-145 could improve glucose uptake.

2.11.6 MicroRNA-29 regulates insulin signaling pathway proteins

Goto-Kakizaki (GK) rats have been used as a non-obese model of Type 2 diabetes. Comparison of skeletal muscle from GK rats and healthy rats based on microRNA arrays identified four up-regulated and eleven down-regulated microRNAs (He et al. 2007). Further work confirmed the miR-29 family was up-regulated in muscle, fat and liver of diabetic rats. Overexpression of the miR-29 family repressed insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The miR-29-induced repression of insulin-stimulated glucose uptake was comparable to that induced by high glucose and insulin treatment (He et al. 2007).

Overexpression of miR-29 in adipocytes was not reported to affect IR tyrosine phosphorylation or cellular content of IRS1. However IRS1 and Akt phosphorylation was decreased in miR-29 expressing adipocytes (He et al. 2007). Inhibition of miR-29 using LNA oligonucleotides activated Akt, but had no effect on glucose uptake (He et al. 2007). Both INSIG1 and CAV2 were confirmed as miR-29 targets based on luciferase assay and Western blots (He et al. 2007), INSIG1 is responsive to insulin and regulates SREBP, while CAV2 is not directly involved in the insulin signaling pathway but has been previously linked to Type 2 diabetes. Other potential miR-29 targets associated with Type 2 diabetes include Syntaxin-1, which is involved in GLUT4 translocation (Cheatham et al. 1996) and transgenic mice over-expressing syntaxin reportedly have elevated insulin-stimulated glucose transport (Spurlin et al. 2004). In the study of He et al. the miR-29 overexpression experiments were all conducted in adipocytes, but it is unknown whether the microRNA:target effects are similar in skeletal muscle. All members of the miR-29 family are highly expressed in muscle, while only miR-29a and miR-29b were confirmed as over expressed in adipose tissue from GK rats. There were eleven more microRNAs identified as over expressed in Type 2 diabetes GK rats, but it is unknown how many of these influence glucose uptake. A future screening study using a microRNA library of inhibitors could identify candidate microRNAs, which regulate muscle glucose uptake.

2.12. Therapeutic possibilities for microRNAs

Following identification of disease-linked microRNAs there are several possibilities for therapeutic intervention. The development of therapeutic interventions has mainly been restricted to cell and mouse models (Mattes et al. 2008). The construction of microRNA mimics has been the focus of much of this research. For example, antisense microRNA oligonucleotides (ASOs) with chemical modifications to improve their stability are reported to reduce liver cholesterol in mice (Krützfeldt et al. 2005). Recently, administration of ASOs targeting miR-320 in mice with cardiac ischemia/reperfusion injury was shown to reduce the size of infarction compared to saline injection (Ren et al. 2009). Other possibilities include using a viral-based approach and infecting a host with a virus containing a microRNA sequence (He et al. 2007). Alternatively, targeting microRNA biogenesis proteins using siRNAs could be used to reduce or block microRNA maturation. Another possibility is targeting microRNA promoters using siRNAs however very few microRNA promoters have been

characterised. Currently, there have been no studies published on the efficacy of microRNA based therapeutic agents in patients, although reportedly clinical trials are underway. However, it is important to firstly understand what microRNAs do and how microRNAs work in skeletal muscle during chronic diseases such as cancer and Type 2 diabetes.

2.13. Summary of research direction

Understanding of microRNA biogenesis, processing and function is advancing at a remarkable pace (Bartel, 2004; Bartel, 2009; Ambros, 2004; Kim et al. 2009). It is clear these small non-coding RNAs can play important regulatory roles in many biological processes (Bushati & Cohen, 2007; Ambros, 2004). There is now huge interest in the role of microRNAs in chronic human diseases including Type 2 diabetes, cardiovascular disease and muscular dystrophies (van Rooij et al. 2008; Chen et al. 2009; Yang & Wu, 2007; Couzin, 2008; Hennessy & O'Driscoll, 2008).

This thesis will focus on determining the role of microRNAs in two chronic diseases affecting human skeletal muscle: Type 2 diabetes characterised by skeletal muscle insulin resistance and cancer cachexia characterised by skeletal muscle wasting. In Type 2 diabetes there is already evidence from diabetic mice suggesting post-transcriptional regulation is important for controlling glucose uptake (Hennessy & O'Driscoll, 2008; He et al. 2007). Genome-wide transcriptome data from our laboratory intriguingly suggests the development of an insulin resistant phenotype could be largely due to post-transcriptional changes (J. Timmons, personal communcation). Microarray profiling of a large Scandinavian Type 2 diabetes cohort (n = 118) found no evidence of transcriptional changes in Type 2 diabetes (Timmons et al. unpublished). Currently, there have been no large microarray studies in cancer cachexia patients. Therefore it is unknown whether there are significant global changes in transcription or whether post-transcriptional regulation is widespread. Nevertheless, to date there have been no studies examining microRNAs in human skeletal muscle from Type 2 diabetes patients or cancer cachexia patients.

Several microRNAs are highly expressed in skeletal muscle including miR-1, miR-133a and miR-206, which are associated with proliferation and differentiation in muscle cell lines (Chen et al. 2006). In mice these microRNAs are modulated in atrophy and hypertrophy models (van Rooij et al. 2008), but it is unknown whether these

microRNAs are modulated in human skeletal muscle *in-vivo*. In Chapter 3, microRNA expression was measured in cancer cachexia patients with varying degrees of weight loss to determine whether microRNAs may provide early biomarkers of cachexia.

In Chapter 4 the thesis focuses on examining the involvement of microRNAs in Type 2 diabetes using a similar approach. Expression of muscle-specific microRNAs was measured in patients with varying degrees of insulin resistance and glucose tolerance. To determine whether microRNAs may be involved in the pathogenesis of Type 2 diabetes, multiple regression was used to examine whether microRNA expression could predict clinical markers of glucose homeostasis and insulin resistance.

In Chapter 5 to determine whether extra-cellular factors may play a role in modulating the expression of disease associated microRNAs, microRNA expression in myotubes was measured in response to insulin and TNFα treatment, which have been previously implicated in cancer cachexia and Type 2 diabetes pathogenesis. In addition, specific microRNA targets were validated in microRNA knockdown experiments.

Chapter 3 - Skeletal muscle microRNAs in cancer cachexia patients

3.1. Introduction

Cachexia has long been recognised as a significant contributing factor to mortality in cancer patients (DeWys, 1985) and over 80% of cancer patients develop cachexia before death (Bruera, 1997). Cachexia is characterised by significant weight loss including both adipose and skeletal muscle tissue. Skeletal muscle protein breakdown in cancer cachexia patients results in functional consequences of fatigue and decreased muscle strength (Bozzetti & Mariani, 2008; Tan & Fearon, 2008). Past reviews have identified systemic inflammation (Durham et al. 2009; McMillan, 2009), plasma cytokines (Seruga et al. 2008), elevated basal energy demands, an acute phase response (Stephens et al. 2008; Skipworth et al. 2007), alteration of muscle growth associated genes, and activation of the ubiquitin-proteolysis pathway in skeletal muscle (Hasselgren & Fischer, 2001; Hasselgren & Fischer, 1997) as central features in cancer cachexia pathogenesis. However, there still remains no consensus on biomarkers for early identification of cachexia in cancer patients (Tan et al. 2008). Furthermore, current therapeutic approaches are primarily limited to managing symptoms of cachexia patients (Bruera, 1997).

The discovery of new biomarkers and understanding the molecular mechanisms involved in cancer cachexia is important to aid early identification of cancer patients at risk of cachexia and to facilitate the development of new therapeutic drugs to combat cachexia (Tan & Fearon, 2008; Tan et al. 2008; Bozzetti & Mariani, 2008). In an attempt to discover new biomarkers and understand the molecular mechanism involved in cancer cachexia, past research has identified transcriptional changes in genes associated with muscle catabolism pathways with potential to cause muscle wasting in animal models (Lecker et al. 2004).

3.1.1 Transcriptional changes in cancer cachexia

It has been suggested common transcriptional changes in gene expression may underlie skeletal muscle atrophy in many systemic diseases such as diabetes, cancer, AIDS and renal failure (Lecker et al. 2004). Although comparison of mRNA expression in skeletal muscle from rats with cancer cachexia, diabetes mellitus, uremia, fasted or paired fed controls revealed that over 90% of mRNAs did not change, a group of genes

termed atrogins were strongly induced across all conditions (Lecker et al. 2004). These included MAFbx and MuRF-1, which are both involved in protein degradation via the ubiquitin-proteasome proteolytic pathway. Recent evidence shows MAFbx can target MyoD for proteolysis during atrophy of myotubes *in-vitro*, as silencing of MAFbx using shRNAi reportedly inhibited MyoD proteolysis (Lagirand-Cantaloube et al. 2009).

The evidence of a common transcriptional program potentially underlying skeletal muscle atrophy characteristic of chronic diseases is limited to rat models of chronic diseases (Lecker et al. 2004). An important caveat to keep in mind when reviewing existing research on cancer cachexia pathogenesis is whether studies have been conducted in cancer cachexia patients or are based on animal cachexia models. For example, FOXO1 has been reported to target MuRF1, MAFbx and myostatin which have been shown to induce cachexia in mice (McFarlane et al. 2006; Zimmers et al. 2002), however there is no supporting evidence of higher FOXO1 in cancer cachexia patients (Schmitt et al. 2007).

It remains to be established whether common transcriptional changes occur in cancer cachexia patients. There is evidence of changes in mRNAs involved in the ubiquitin-proteasome pathway. For example, significantly higher ubiquitin and 20S proteasome subunit mRNA expression is reported in skeletal muscle from cancer patients (Williams et al. 1999). In contrast, a study in a larger group of lung cancer patients reported no change in mRNA of ubiquitin-proteasome pathway genes (Jagoe et al. 2002). Yet in gastric cancer patients ubiquitin mRNA expression was found to be higher than in control patients (Bossola et al. 2003). When proteasome sub-unit expression was compared in patients with different cachexia severity, there appeared to be no evidence of changes in proteasome sub-unit mRNA in patients with less than 10% weight loss (Khal et al. 2005). Taken together these studies suggest ubiquitin-proteasome gene expression is higher in cancer cachexia patients, but does not explain early protein degradation in cachexia. Therefore, further studies are needed to identify early biomarkers of cachexia onset before >10% weight loss occurs.

Systemic overexpression of myostatin in adult mice via activation of serum myostatin has been reported to induce significant muscle and fat loss comparable to human cachexia (Zimmers et al. 2002). Mice injected with a Chinese hamster ovary (CHO) cell line with inducible myostatin expression lost on average 33% body weight

(Zimmers et al. 2002). There do not appear to be any studies reporting myostatin expression in cancer cachexia patients. However, patients with atrophy of type 2 non-oxidative muscle fibres have been shown to have elevated myostatin protein, but unchanged myostatin mRNA (Wójcik et al. 2008). It remains to be seen whether myostatin is a biomarker of cachexia. The lack of correlation between myostatin mRNA and protein levels in patients with skeletal muscle atrophy indicates that myostatin could be regulated post-transcriptionally. This is supported by evidence that a mutation in the myostatin 3'UTR in TEXEL sheep creates a miR-206 binding site which leads to a significant increase in muscularity via post-transcriptional suppression of myostatin (Clop et al. 2006). To date no studies have examined microRNA expression in skeletal muscle of cancer cachexia patients, despite microRNAs representing an important group of global post-transcriptional gene regulators.

3.1.2 Possible changes in muscle-specific microRNAs in cancer cachexia

The muscle-specific microRNAs, miR-1, miR-133a and miR-206 have all been hypothesised to play a central role in muscle degeneration (Chen et al. 2006; van Rooij et al. 2008; McCarthy et al. 2007; Rosenberg et al. 2006; Kim et al. 2006), but to date there is very limited data from skeletal muscle biopsies of patients with muscle degeneration. Early during differentiation of skeletal muscle myoblasts there is consistent evidence that miR-1, miR-133a and miR-206 increase dramatically (Chen et al. 2006; Kim et al. 2006). In our laboratory miR-1, miR-133a and miR-206 have been observed to continue to increase even after initial formation of myotubes (Brzeszczynska et al. under revision). Furthermore, miR-1, miR-133a and miR-206 are potentially regulated by myogenic differentiating factors (Rao et al. 2006; Liu et al. 2007). For example, MyoD, Mef2 and SRF binding sites have been identified upstream of the genes encoding miR-1, miR-133a and miR-206 (Zhao et al. 2005; Liu et al. 2007; Rao et al. 2006; Sweetman et al. 2008). The presence of common transcription factor binding sites suggests that transcription of miR-1, miR-133a and miR-206 genes may be activated in unison (Chen et al. 2006; van Rooij et al. 2008; McCarthy, 2008). The evidence showing increased miR-1, miR-133a and miR-206 transcription and mature miR-1, miR-133a and miR-206 abundance during skeletal muscle differentiation *in-vitro* suggests these microRNAs may also have important regulatory functions in adult muscle growth and regeneration *in-vivo* (Chen et al. 2006; Rao et al. 2006; Brzeszczynska et al. under revision).

Studies on miR-1, miR-133a and miR-206 during muscle hypertrophy and atrophy have been mostly limited to *in-vivo* mouse models (McCarthy & Esser 2007; McCarthy et al. 2007; Carè et al. 2007). In a mouse model of cardiac hypertrophy, down-regulation of miR-133a was observed leading to heart failure (Carè et al. 2007). The cardiac hypertrophy occurring in heart failure is associated with an increase in fibrosis and extracellular matrix proteins and a decline in contractile function regulated by miR-133a down-regulation (Pretorius et al. 2008). However, it is not known whether miR-133a down-regulation in skeletal muscle leads to similar functional changes in fibrosis and extracellular matrix proteins. Counter-intuitively, miR-133a and miR-1 have been reported to be down-regulated in skeletal muscle of mice after 7 days of functional overload (McCarthy and Esser, 2007). This suggests miR-133a may be regulating positive functional changes in skeletal muscle while regulating negative functional changes in cardiac muscle.

Skeletal muscle atrophy induced by unloading during space flight is reported to be associated with a significant down-regulation of miR-206 (Allen et al. 2009), although no other microRNAs were measured. Reduced physical activity and unloading during bed-rest are associated with later stages of cancer cachexia (Fouladiun et al. 2007; Dahele et al. 2007; Moses et al. 2004; Makridis et al. 1997). Therefore, microRNAs modulated during unloading and physical inactivity could be relevant in cancer cachexia. However, the evidence of miR-206 expression changes during atrophy appears to be conflicting, with reports of both up- and down-regulation of miR-206 (McCarthy, 2008; McCarthy et al. 2007; Yuasa et al. 2008; Allen et al. 2009). In mdx mice, an animal model of muscular dystrophy, miR-206 expression is reported to be significantly elevated in the diaphragm but not hindlimb muscle (McCarthy et al. 2007). A later study on mdx mice found elevated miR-206 expression in the tibialis anterior muscle, while no differences in miR-1 or miR-133a were observed (Yuasa et al. 2008). There is some evidence that dystrophin may be decreased in skeletal muscle from gastro-intestinal cancer patients similar to mdx mice (Acharyya et al. 2005). Therefore, changes in microRNAs in the mdx mouse could be relevant in cancer cachexia patients, although microRNA changes appear to be dependent on the atrophy model used.

The evidence from muscular dystrophy patients does not support the idea of a central role of miR-1, miR-133a and miR-206 in muscle atrophy (Eisenberg et al. 2007). For example, in skeletal muscle from patients with a range of muscular dystrophies

characterised by reduced muscle mass, miR-1, miR-133a and miR-206 expression was unchanged (Eisenberg et al. 2007). This suggests miR-1, miR-133a and miR-206 may not be a central characteristic of muscle wasting associated with muscular dystrophies in adult patients. However, the microRNA arrays used by Eisenberg et al. were custom manufactured and there is no published data on the reliability or validity of these microRNA arrays (Ach et al. 2008; Liu et al. 2008). Eisenberg et al. reported microRNA array validation data, but there appears to be limited agreement between microRNA array and RT-qPCR microRNA expression data, whether this was due to variation in the RT-qPCR data or microRNA array data is unknown. In other studies microRNAs have been quantified by RT-qPCR using stem-loop primers, which have been subject to extensive validation studies (Chen et al. 2005). So until more microRNA expression data are available from muscular dystrophy patients or the microRNA array platform is more widely used such findings must be interpreted cautiously.

3.1.3 Possible changes in miR-21 in cancer cachexia

The presence of systemic inflammation in cancer cachexia patients suggests microRNAs associated with inflammation may also be altered in cachexic muscle (McMillan 2009; Skipworth et al. 2007). Recently it was reported there was a trend for an elevation of mature miR-21 expression in skeletal muscle from intensive care unit (ICU) patients and microarray data indicated precursor miR-21 was significantly up-regulated (Fredriksson et al. 2008). Skeletal muscle loss occurs during sepsis in ICU patients with multiple organ failure and the substantial loss of muscle mass can lead to prolonged recovery. Surprisingly, in skeletal muscle from ICU patients, mitochondrial protein synthesis was maintained (Fredriksson et al. 2008). However, ICU patients were being treated with insulin, which is known to stimulate protein synthesis (Pearlstone et al. 1994; Lundholm et al. 2007). The predicted targets of miR-21 were reported to include genes involved in the ubiquitin-proteolysis pathway and the JAK/STAT pathway (Fredriksson et al. 2008). Evidence from studies on miR-21 in cancer tumours, suggests it can regulate cell survival pathways. However, microRNA targeting is reported to be context specific (Bartel, 2009), therefore it is uncertain whether miR-21 functions demonstrated in other cell types are relevant to skeletal muscle. In patients with various muscular dystrophies miR-21 was consistently up-regulated (Eisenberg et al. 2007). Thus the existing evidence suggests miR-21 may be a plausible candidate as an early biomarker of cancer cachexia.

3.1.4 Identifying microRNA biomarkers in cancer cachexia

The advances in microRNA array technology have led to many studies attempting to identify potential microRNA biomarkers of disease pathogenesis. For example, microRNA array expression data has been used to classify different tumour types (Blenkiron et al. 2007; Lu et al. 2005; Dixon-McIver et al. 2008; Szafranska et al. 2008). MicroRNA arrays could potentially be able to identify cancer patients at risk of developing cachexia before significant weight loss and deterioration in quality of life. MicroRNA arrays are particularly advantageous as expression levels of all known microRNAs can be measured simultaneously and thus possible microRNA biomarkers to follow-up can be identified rapidly, compared to alternative approaches such as Northern blot or RT-qPCR (Clancy et al. 2007; Liu et al. 2008; Chen et al. 2005; Válóczi et al. 2004). However, it remains imperative that microRNA array expression data is validated independently using an alternative technology for detection of microRNAs (Ach et al. 2008).

The existing evidence strongly suggests microRNAs may contribute to cancer cachexia pathogenesis and therefore may represent possible early biomarkers of cachexia in cancer patients. Specific muscle microRNAs, miR-1, miR-133a, miR-133b and miR-206 are strongly induced during skeletal muscle differentiation, altered during hypertrophy and atrophy in mice (Chen et al. 2006; Nakajima et al. 2006; Boutz et al. 2007; Yuasa et al. 2008; McCarthy et al. 2007; Kim et al. 2006; Rosenberg et al. 2006). Recently, miR-21 was identified as a possible modulator of muscle wasting in ICU patients and up-regulated in muscular dystrophy patients (Fredriksson et al. 2008; Eisenberg et al. 2007). Therefore, miR-1, miR-133a, miR-133b, miR-206 and miR-21 were hypothesised to be potential early biomarkers of cachexia. Further novel microRNA biomarkers were identified through experimental validation of microRNA array data from cancer cachexia patients. Evidence supporting the function of candidate cachexia microRNA biomarkers was examined using microarray data from cancer cachexia patients, to determine whether there were global changes in the expression of microRNA targets.

3.1.5 Aims

- Identify muscle microRNA biomarkers of cachexia severity in cancer patients.
- Examine muscle microRNA target expression for evidence of microRNA function in cancer cachexia patients.

3.2. Methods

Cancer cachexia patients were recruited as part of a wider cross-sectional study to discover new molecular and functional biomarkers associated with cachexia severity in collaboration with Prof Fearon and the Cancer cachexia group at Edinburgh University, UK. I was specifically involved in examining the role of microRNAs in cancer cachexia. Patients had a diagnosis of upper gastrointestinal cancer (oesophageal, gastric, pancreatic) and were undergoing surgery for resection of primary tumour. Healthy, weight stable patients undergoing surgery for benign conditions (e.g. incisional hernia repair) were included as controls. All patients had the study information explained to them by a clinician and gave their informed consent before clinical examination. Patients' weight loss was calculated based on measurement of body mass during initial clinical examination and patients' estimated body mass before cancer diagnosis. In the controls body mass was measured during clinical examination and no weight loss was reported. Mid arm muscle circumference (MAMC) and C-reactive protein (CRP) were both measured. BMI was calculated as body mass (kg) / body height $(m)^2$ based on estimated body mass before cancer diagnosis (pre-BMI) and patients' body mass at clinical examination (post-BMI). Muscle biopsies were taken from the rectus abdominis during surgery, which involved removing a 1 cm^3 section of muscle by sharp dissection while the rectus abdominis was exposed. The cross-sectional cancer cachexia study received REC approval (06/S1103/75) from the Lothian Regional Ethics Committee, Scotland.

3.2.1 Approach

Firstly, the focus was on determining whether the muscle specific microRNAs, miR-1, miR-133a, miR-133b and miR-206 were differentially expressed in skeletal muscle biopsies from control (0% weight loss), pre-cachexia (0-10% weight loss) and cachexia (>10% weight loss) patients. Secondly, following up from previous work by colleagues in our laboratory on miR-21 expression in skeletal muscle from ICU patients (J. Timmons, personal communication), elevated pri-miR-21 in the ICU patients was confirmed, then primary and mature miR-21 expression was measured in the cancer cachexia patients. Thirdly, microarray data from cancer-cachexia patients (I. Gallagher, personal communicaton) was used to examine changes in microRNA target expression. Fourthly, further potential microRNA biomarkers were measured using RT-qPCR to follow-up preliminary findings from microRNA array profiling (I. Gallagher, personal communication) as part of experimental validation. Finally, gene ontology and pathway

enrichment analysis were used to explore the function of predicted targets of microRNAs changed in cancer cachexia.

3.2.2 Clinical characteristics of the cancer cachexia patient cohort

MicroRNA expression was analysed in 58 patients classified into three groups, cachexia pre-cachexia or control based on definitions provided by the SCRINO international cancer cachexia working group (Bozzetti & Mariani, 2008). The cachexia group included all patients with above 10% weight loss (n = 19; males = 8, females = 11). The pre-cachexia group included all patients with between 0-10% weight loss (n = 32; males = 24, females = 8). The control group included patients without cancer and with no weight loss (n = 7; males = 5, females = 2). The patient characteristics for each group are shown in Table 3-1. Age was significantly lower in the control group compared to the pre-cachexia and cachexia groups ($P < 0.05$). BMI (at resection of primary tumour) and %weight loss were significantly lower in cachexia group compared to controls ($P < 0.05$). Plasma CRP was significantly higher in the cachexia group compared to the pre-cachexia group ($P < 0.05$). Ten patients were undergoing chemotherapy (n = 5 pre-cachexia; n = 5 cachexia).

Table 3-1. Patient characteristics

Patient characteristics	Control (n=7) 0% weight loss	Pre-cachexia (n=32) 0-10% weight loss	Cachexia (n=19) >10% weight loss
Age (y)	51 ± 15	67 ± 10*	64 ± 9*
BMI	30.6 ± 3.4	26.6 ± 2.6	23.6 ± 4.4*
%Weight loss	0.0 ± 0.0	4.11 ± 2.9	16.22 ± 7.2* †
CRP (mg/l)	2.4 ± 1.8	8.3 ± 16.2	30.3 ± 43.8 †
MAMC (cm)	25.9 ± 3.2	24.7 ± 2.6	23.9 ± 3.0

Data show mean ± SD. * $P < 0.05$ compared to control. † $P <$ compared to pre-cachexia.

3.2.3 RNA isolation

Patient muscle biopsy samples were prepared whilst on liquid nitrogen; 15-20 mg tissue was cut from each sample. Tissue was homogenised in 1 ml of TRIzol for 15 s using a mini-beadbeater. Samples were then incubated for 5 min at 25°C. 200 µl chloroform was added, vortexed for 15 s, then incubated for 3 min at 25°C. Samples were then centrifuged at 12000g for 15 min at 4°C. The aqueous phase was extracted and 500 µl isopropanol was added. Samples were then incubated for 10 min at 25°C and

centrifuged at 12000g for 10 min at 4°C. The suspension was discarded and 1 ml 70% EtOH was added, followed by centrifugation at 7500g for 5 min at 4°C. The suspension was again discarded and the pellet air-dried for 2 min. The RNA pellet was dissolved in 15 µl DEPC water. Samples were stored at -80°C prior to further analysis.

3.2.4 Determination of RNA concentration and quality

RNA concentration and quality was determined on RNA Nano chips using an Agilent 2100 Bioanalyser. The RNA Nano chip uses micro capillary electrophoresis to separate samples and RNA is detected via laser-induced fluorescence (Fleige & Pfaffl, 2006). It is advantageous as it is sensitive to RNA concentrations down to 200 pg. The RNA Nano chips were run according to the manufacturer's instructions. Firstly, a gel-dye mix was prepared using 1 µl RNA 6000 Nano dye and 65 µl Agilent RNA 6000 Nano gel matrix. This was centrifuged for 10 min at 1500g and stored in the dark at 4°C. The RNA Nano chip was primed with 9 µl gel-dye mix using a syringe. Then 5 µl RNA 6000 Nano marker was pipette into the sample wells and the ladder well. RNA samples were prepared by heating on a heating block for 2 min at 70°C, cooling on ice for 2 min followed by centrifugation at 1000 g for 2 min. The RNA Nano chip was then loaded with 1 µl sample per well and 1 µl RNA 6000 Nano ladder in the ladder well. The RNA Nano chip was vortexed for 60 s at 2400 rpm and then run on the Agilent 2100 Bioanalyser.

The Agilent 2100 software determines a RIN score for each sample based on an algorithm taking into account the shape of the electropherogram (Fleige & Pfaffl, 2006). RIN scores of 10 are optimal and represent high quality RNA with limited RNA degradation (Schroeder et al. 2006). The RNA concentration is determined based on the area under the curve for 18S and 28S. Many of the initial RNA samples had RIN scores of less than 7 and the gels showed evidence of RNA degradation (Supplementary Figure 3-1). The Agilent Bioanalyser 2100 is reported to be more susceptible to novice operator variation compared to other spectrophotometer methods of RNA quantification such as the Nanodrop (Ar et al. 2009). To ensure operator error was not the cause of poor RIN scores, samples with poor RIN scores were re-run on the RNA Nanochips. There was evidence of RNA degradation in a significant proportion of the samples during early RNA isolation (data not shown). This may have been the result of freezer breakdown affecting some muscle biopsy samples, as poor sample handling may lead to RNA degradation (Bustin & Nolan, 2004), but there is no data on which samples may

have been involved. Nevertheless, the initial microRNA RT-qPCR analysis was conducted on n = 58 patient samples which all had acceptable RIN scores above seven.

3.2.5 Mature-microRNA reverse transcription

Reverse transcription (RT) of RNA to cDNA was conducted using the TaqMan® MicroRNA Reverse Transcription Kit according to the manufacturer's instructions. The RT uses microRNA specific stem-loop primers. These first hybridise at the 3' end of microRNA molecules and are then reverse transcribed using MultiScribe reverse transcriptase (Chen et al. 2005). Therefore closely related microRNAs that differ by a single nucleotide can be quantified specifically (Chen et al. 2005). Each RT reaction was set-up to contain between 2-10 ng of total RNA. Reactants were thawed on ice and RT reactions were prepared. Each RT reaction contained 0.15 µl 100 mM dNTPs, 1 µl MultiScribe Reverse Transcriptase [50 U/µl], 1.5 µl Reverse Transcription Buffer, 0.19 µl RNase Inhibitor [20 U/µl] and 4.16 µl Nuclease-free water. To each RT reaction 5 µl RNA was added, followed by 3 µl of RT primer. Samples were mixed gently, centrifuged ~3 s at 0.5 RPM then incubated on ice for 5 min. The reverse transcription was performed on a thermal cycler programmed to run for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C then held at 4°C. cDNA samples were kept at 4°C and analysed within 24 h.

3.2.6 Real-time quantitative PCR of mature-microRNAs

TaqMan® MicroRNA Assays were used to measure the microRNAs listed in Table 3-2 according to the manufacturer's instructions. Each PCR reaction was calculated to contain 1 µl TaqMan MicroRNA Assay [20x], 1.33 µl cDNA, 10 µl Taqman 2 x universal PCR master mix [No AmpErase UNG] and 7.67 µl nuclease-free water. For each microRNA all patient PCR reactions were prepared in triplicate on the same 384-well plate, triplicate no template controls (NTC) were also run. The plate was sealed and spun down at 3000 rcf in a centrifuge at 4°C. The PCR reaction was run on an Applied Biosystems 7300/7500 Fast Real-Time PCR system in 9600 emulation mode. The thermal cycling consisted of a 10 min hold at 95°C, then 40 cycles of 15 s at 95°C and 60 s at 60°C. The Ct threshold was manually determined during the exponential fluorescence phase. Ct values for triplicates were averaged, and ΔCt values computed using RNU48, which is a small nucleolar RNA, as the endogenous control. RNU48 was found not to vary significantly between cachexia, pre-cachexia and control groups (Supplementary Figure 3-3). Fold change was calculated using the -2ΔΔCT method

(Schmittgen & Livak, 2008). Taqman microRNA assays are reported to detect changes in microRNA abundance over seven orders of magnitude (Chen et al. 2005). Standard curves were prepared for miR-133a, miR-206 and miR-1 based on five cDNA dilutions (data not shown). The standard curve was linear across the range of cDNA dilutions in agreement with previous studies (Chen et al. 2005). Furthermore, the Taqman microRNA assays appeared to specifically detect mature microRNA sequences, as there was no evidence of pri-microRNA or pre-microRNA detectable in RT-qPCR products run on an agarose gel (Supplementary Figure 3-2).

Table 3-2. Mature microRNA sequences

MicroRNA	miRbase ID	Mature microRNA Sequence
Hsa-miR-1	MIMAT0000465	5'-uggaauguaaagaaguaugua-3'
Hsa-miR-133a	MIMAT0001475	5'-uuggucccuucaaccagcugu-3'
Hsa-miR-133b	MIMAT0000770	5'-uuggucccuucaaccagcua-3'
Hsa-miR-206	MIMAT0000879	5'-uggaauguaaggaagugugugg-3'
Hsa-miR-21	MIMAT0000076	5'-uagcuuaucagacugauguuga-3'
RNU48	NR_002745	5'-gaugaccccagguaacucugagugug ucgcugaugccaucaccgcagcgcucugacc-3'

3.2.7 Design and validation of pri-miR-21 primers

To determine whether pri-miR-21 transcription was regulated in cachexia and to confirm pri-miR-21 was regulated in sepsis patients, primers were designed to amplify the intronic regions between the pre-microRNA hairpin and the predicted host gene (Figure 3-1). All primers for pri-microRNA transcripts are listed in Table 3-3. Primers were obtained from Invitrogen UK. Primer efficiency was tested on five serial dilutions of cDNA using the RT-qPCR protocol described in section 4.2.7 and is shown in Supplementary Figure 3-4.

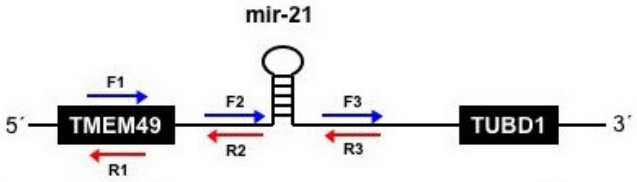


Figure 3-1. Location of primers designed to amplify pri-miR-21. Primers were also designed to amplify the predicted host gene TMEM49. Arrows indicate location of forward and reverse primers.

Table 3-3. Primer sequences to amplify pri-miR-21.

Target	Identifier		Primers, sequences or probes
TMEM49	ENSG000	F1	5'- gcagaagccatttcaggagt -3' (forward)
(host gene)	00062716	R1	5'- tcaaacatccaggacaacca -3' (reverse)
TMEM49 –	ENSG000	F2	5'- gtcagaatagaatagaattggggttc -3' (forward)
Pri-hsa-miR-21	00199004	R2	5'- aaggtggtacagccatggag -3' (reverse)
Pri-hsa-miR-21	ENSG000	F3	5'- tgaataaaatccctaagaagactgc -3' (forward)
–TUB1	00199004	R4	5'- aagaagagacaaagttgtcgatacag -3' (reverse)

Primers were obtained from Invitrogen, UK.

3.2.8 Pri-miR-21 reverse transcription and real-time quantitative PCR

RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK) and is described in detail in Chapter 4 in section 4.2.8. Following reverse transcription cDNA samples were stored at -20°C until further analysis. SYBR green reagents (Applied Biosystems, UK) were used for RT-qPCR reaction to amplify regions spanning pri-miR-21 transcript; the primers used are listed in Table 3-3. The preparation of each RT-qPCR reaction is described in detail in Chapter 4 in section 4.2.9. The PCR reaction was run on an Applied Biosystems 7300/7500 Fast Real-Time PCR system in standard mode. Ct values for triplicates were averaged, and ΔCt values computed using 18S as the endogenous control. Fold change was calculated using the -2ΔΔCT method (Schmittgen & Livak, 2008).

3.2.9 MicroRNA array experimental validation

Cachexia patient RNA samples were prepared as described in section 3.2.3-3.2.4. Following identification of possible phenol contamination in Type 2 diabetes patient samples described in section 4.2.5 it was decided to reprecipitate cachexia patient RNA samples, thus cachexia patient RNA samples (n = 58) were re-quantified using the

Nanodrop spectrophotometer (Thermoscientific, UK). The procedure for RNA quantification using the Nanodrop spectrophotometer is described in section 4.2.5. Patient characteristics are shown in Supplementary Table 3-2. The microRNA arrays were conducted by colleagues on pooled cachexia patient RNA and analysed using Significance Analysis of Microarray (SAM) in R to determine differentially expressed microRNAs (I. Gallagher, personal communication) and hence is not presented herein. Experimental validation of the microRNA array data was conducted on 7 up-regulated microRNAs (miR-23a, miR-27b, miR-29a, miR-29b, miR-143, miR-195, miR-424) and 1 down-regulated microRNA (miR-208) using RT-qPCR as described in section 3.2.5 and 3.2.6. The mature microRNA sequences are listed in Table 3-4.

Table 3-4. Mature microRNA sequences for microRNA array validation.

MicroRNA	miRbase ID	Mature microRNA Sequence
Hsa-miR-23a	MIMAT0000078	5'-aucacauugccagggauuucc-3'
Hsa-miR-27b	MIMAT0000419	5'-uucacaguggcuaaguucugc-3'
Hsa-miR-29a	MIMAT0000086	5'-uagcaccaucugaaaucgguua-3'
Hsa-miR-29b	MIMAT0000100	5'-uagcaccauuugaaaucaguguu-3'
Hsa-miR-143	MIMAT0000435	5'-ugagaugaagcacuguagcuc-3'
Hsa-miR-195	MIMAT0000461	5'-uagcagcacagaaauauuggc-3'
Hsa-miR-424	MIMAT0001341	5'-cagcagcaauucauguuuugaa-3'
Hsa-miR-208	MIMAT0000241	5'-auaagacgagcaaaaagcuugu-3'

3.2.10 MicroRNA target prediction and expression analysis

The binding of microRNA to target mRNAs occurs between the "seed" region of the microRNA (nucleotide 2-7 of the 5`end of the mature microRNA) and the 3`UTRs of the mRNAs. Genes predicted to be regulated by specific microRNAs were obtained using TargetScan 4.2 (Lewis et al. 2003). The mean absolute expression approach (Arora and Simpson, 2008) was used to determine whether there were detectable shifts in the average expression of microRNA targets in skeletal muscle from cancer cachexia patients when *in-vivo* microRNA expression was changed. Unpublished microarray data were available from our laboratory (I. Gallagher, personal communication; Stephens et al. manuscript under revision) from n = 20 cancer cachexia patients included in the present study. R and Bioconducter were used to process the data, CEL files were normalized using the MAS 5.0 algorithm with the simplyaffy package in

Bioconducter. Present/absent calls across all chips were computed and absent probes were removed. Using bioMart the present called probes were mapped to HUGO gene symbols from Ensembl and average gene expression computed for each patient. Microsoft Access database was used to link microRNA targets with gene expression data. For each patient, average expression of targets for each cachexia microRNA biomarker was computed. To determine whether there were any significant detectable shifts in microRNA target expression, differences in average microRNA target expression between cachexia, pre-cachexia and control groups were analysed using one-way ANOVA.

3.2.11 Gene ontology and pathway enrichment analysis

Gene ontology (GO) analysis (Ashburner et al. 2000) was used to examine the functions of microRNA targets. To determine which GOs may be regulated by microRNAs changed in cancer cachexia patients The Expression Analysis Systematic Explorer (EASE) (Hosack et al. 2003) was used. EASE conducts a statistical analysis of GO terms associated with a list of genes (i.e. microRNA targets) and determines which GO terms are the most over-represented compared to the background of all genes represented in the human genome. The GO analysis was based on GO terms within three GO classifications, Molecular Function, Cellular Component and Biological Process. EASE calculates GO terms over-represented using the EASE score, which is a modified version of Fishers Exact probability. The Fisher exact probability is calculated based on the probability of the "List Hits" in the "List Total" given the frequency of "Population Hits" in the Population Total". The "Population total" refers to all genes annotated within the specified GO classification. "Population hits" refers to the number of genes associated with a given GO term in the population of genes annotated within the specified GO classification. "List Hits" refers to the number of genes within the gene list (i.e. microRNA target list) that are associated with a given GO term. The EASE score is the upper bound of the distribution of jackknifed Fisher exact probabilities based on 500 iterations, which helps adjust the significance of GO terms with only a few genes compared to GO terms with many associated genes. EASE also computes an FDR, to indicate the percentage of GO terms enriched by chance. The FDR provides the probability of observing a given enriched GO term from a given gene list by chance. The FDR was computed from multiple iterations (n = 500) on random gene lists from the population. It is important to emphasise that the GO analysis is an exploratory tool to discover the underlying biology of a list of genes, therefore there are

no specific p-value cut-offs. Instead the over-representated GO terms were ranked based on an FDR of less than 10%. All GO analysis results are tabulated in the appendix.

The pathway analysis was conducted using EASE as described previously to search for microRNA targets over represented in pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000). The results are graphically presented showing the number of microRNA targets enriched or over-represented in a given pathway ranked by FDR. No specific *P* value cut-offs were specified, but results are presented when FDR was less than 10%. It is important to highlight that the pathway analysis is exploratory, to indicate if a microRNA may co-ordinately target specific signaling pathways relevant to cancer cachexia. KEGG pathways are based on the current knowledge of genes associated with signaling pathways, therefore microRNA targets which may be relevant to disease pathogenesis may not be considered if KEGG pathway enrichment is considered alone, hence the approach of using both GO and pathway enrichment analysis.

3.2.12 Statistical Analysis

Statistical analysis was conducted in Prism v 5.0 (GraphPad). Expression of mature miR-1, miR-133a, miR-133b, miR-206 was compared between cachexia, pre-cachexia and control groups (n = 58) using one-way ANOVA assuming equal variances between groups based on Bartlett's test. In addition, expression of primary and mature miR-21 was compared in pancreatic cancer cachexia patients, ICU patients and controls using one-way ANOVA. Post-hoc Bonferroni tests were performed where necessary to identify inter-group differences. When the assumption of equal variances was not met the non-parametric Kruskal Wallis test was run.

The classification of patients into discrete groups (cachexia, pre-cachexia and control) does not take into account that weight loss or cachexia severity is a continuous variable. Therefore another approach was taken; linear regression was used to test whether individual patient microRNA expression could explain a significant proportion of the variance in weight loss. To eliminate any additional variation associated with cachexia development in different cancer types, further linear regression was conducted on the pancreatic cancer cachexia patients only (n = 19) to determine whether cancer cachexia

severity was associated with microRNA expression specifically in pancreatic cancer patients.

The experimental validation of microRNA arrays was conducted on a smaller patient cohort (n = 20), differences in expression of miR-23a, miR-27b, miR-29a, miR-29b, miR-143, miR-195, miR-208 and miR-424 between cachexia, pre-cachexia and control patients were compared using one-way ANOVA. Data is presented as mean ± SE unless otherwise stated. $P < 0.05$ was considered as significant.

3.3. Results

3.3.1 Muscle microRNA expression in cancer cachexia patients

Several microRNAs are abundantly expressed during skeletal muscle differentiation, yet their role in adult skeletal muscle is unknown. The expression of mature miR-1, miR-133a, miR-133b and miR-206 was measured in skeletal muscle biopsies from cancer patients with pre-cachexia or cachexia and compared to controls.

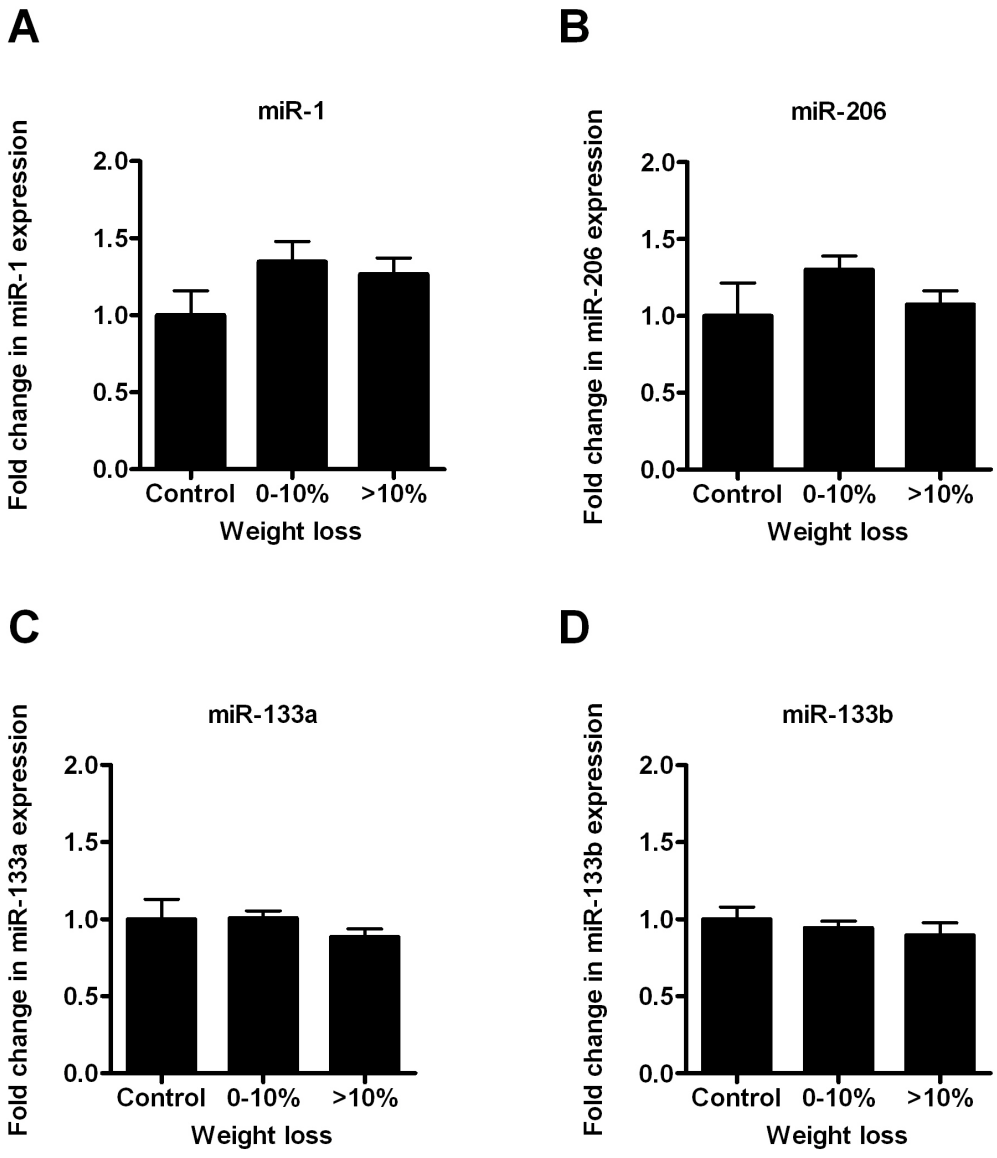


Figure 3-2. Fold change in (A) miR-1, (B) miR-206, (C) miR-133a, and (D) miR-133b expression in skeletal muscle from cachexia patients with >10% weight loss (n = 19), pre-cachexia patients with 0-10% weight loss (n = 32), and control patients (n = 7). Data normalised to control and presented as mean ± SE.

Figure 3-2 shows the fold change in miR-1, miR-133a, miR-133b and miR-206 expression in pre-cachexia and cachexia patients compared to controls. No significant differences were detected. The lack of significant differences in muscle miR-1, miR-133a, miR-133b and miR-206 expression between controls, pre-cachexia and cachexia patients may have been due to the grouping criteria.

To examine this further, miR-1, miR-133a, miR-133b and miR-206 expression was plotted against weight loss (Supplementary Figure 3-5). Bivariate correlations were used to examine whether there was evidence of a linear association between patient weight loss and miR-1, miR-133a, miR-133b and miR-206 expression. There appeared to be only weak correlations between microRNA expression and %weight loss among cancer patients with various tumours including pancreatic, oesphagus, gastric and oesophagogastric junction (OGJ) (Supplementary Figure 3-5). This may have been due to variability in microRNA expression among cancer patients with minimal weight loss since diagnosis.

An alternative explanation was that different cancer types might influence microRNA expression during cachexia development, which would explain the lack of a clear association between muscle growth-linked microRNAs and %weight loss. In the present cancer cachexia cohort, around 30% patients had pancreatic tumours (Supplementary Table 3-1). The incidence of cachexia is reported to be higher in pancreatic cancer patients (Fearon, 1992), although it has not yet been establish why there is a higher incidence of cachexia among pancreatic cancer patients. Therefore, miR-1, miR-133a, miR-133b and miR-206 expression was re-analysed focusing on the pancreatic cancer patients only (n = 19; Supplementary Table 3-1).

3.3.2 Muscle microRNA expression predicts weight loss in pancreatic cancer

The association between miR-1, miR-133a, miR-133b, miR-206 and weight loss was examined in pancreatic cancer patients (n = 19) with varying weight loss and controls with no weight loss (Figure 3-3). Multiple linear regression was used to examine whether there was a significant relationship present.

Table 3-5. Multiple regression model of muscle specific microRNA expression as a predictor of weight loss in pancreatic cancer cachexia patients.

Variable	Coefficient	T ratio	*P*-value
Constant	127.4 ± 28.0	4.6	<0.001
miR-1	-2.9 ± 4.3	0.7	0.52
miR-206	5.8 ± 3.8	1.5	0.15
miR-133a	16.7 ± 5.0	3.3	0.005
miR-133b	1.5 ± 4.5	0.3	0.74

A multiple linear regression model including miR-1, miR-133a, miR-133b and miR-206 explained 66% of the variance in weight loss ($F = 6.7$, $P = 0.003$; Table 3-5). Thus lower miR-1, miR-133a, miR-133b and miR-206 expression was associated with higher weight loss in pancreatic cancer patients (Figure 3-3). The microRNA expression data was normally distributed, there was no evidence of multicollinearity (VIF<0.75) and the residuals appeared to be randomly distributed (Supplementary Figure 3-6). However, only miR-133a was a significant contributer to the regression model ($t = 3.3$, $P = 0.005$), which suggest miR-1, miR-206 and miR-133b expression data were not essential and a simpler regression model based on miR-133a alone may well predict weight loss.

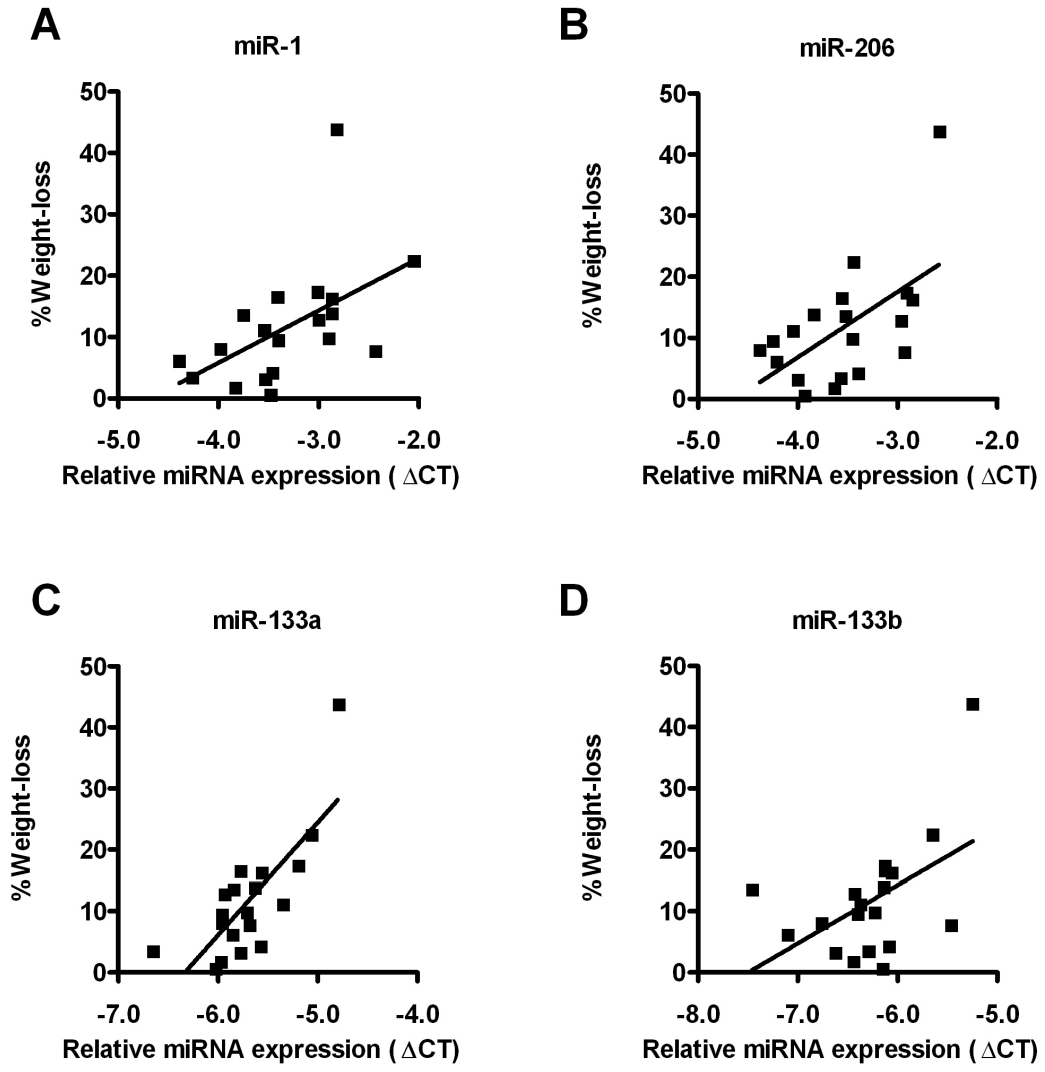


Figure 3-3. (A) miR-1, (B) miR-206, (C) miR-133a and (D) miR-133b expression correlates with weight loss in pancreatic cancer patients. Higher ΔCT indicates lower microRNA expression.

3.3.3 Expression of microRNA biogenesis genes in cancer cachexia patients

Regulation of microRNA biogenesis gene and protein expression can lead to global effects on mature microRNA expression. Expression of the microRNA biogenesis genes DROSHA, DGCR8, DICER1 and AGO2 was determined from patients' microarray data (n = 20). However, there were no significant differences in DROSHA, DGCR8, DICER1 or AGO2 expression between controls, pre-cachexia and cachexia patients (Figure 3-4).

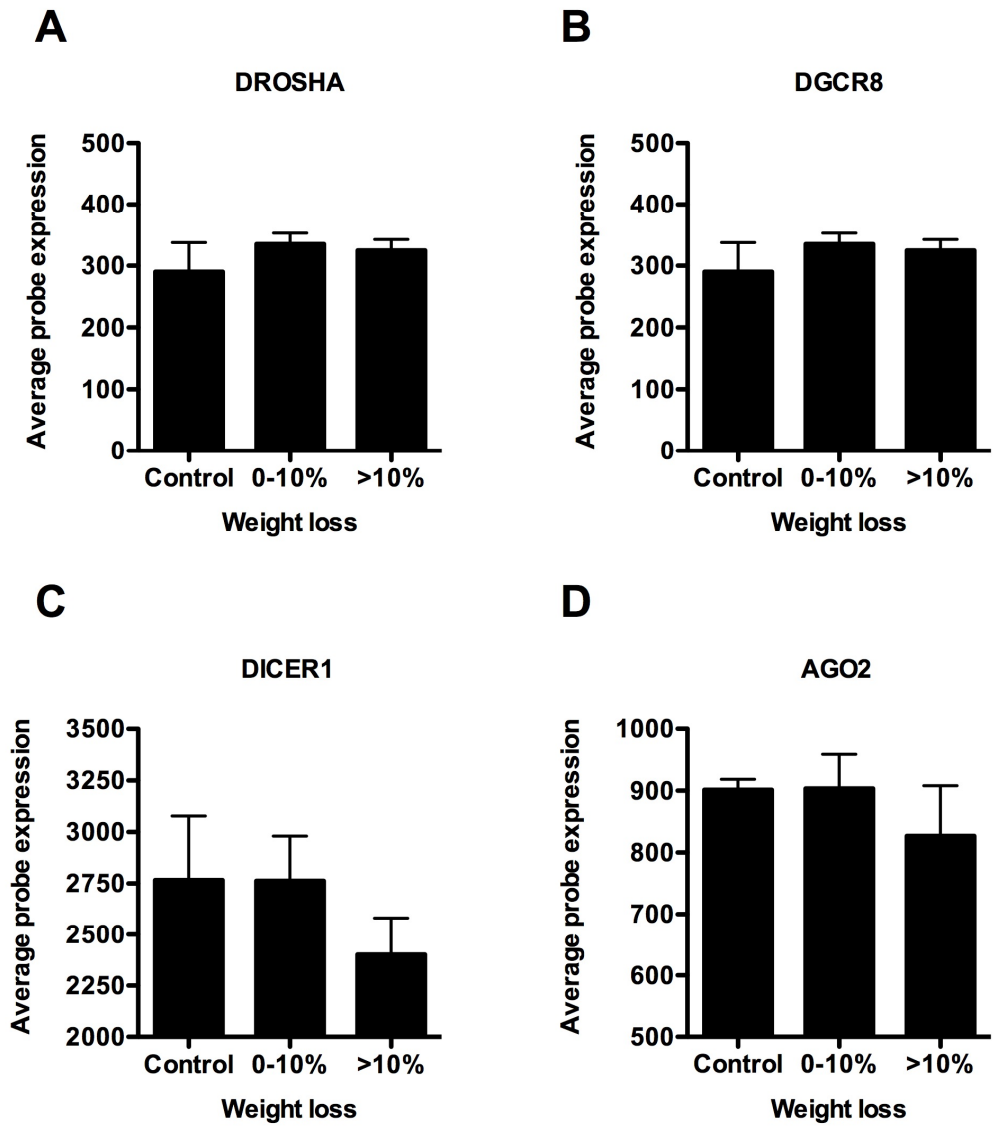


Figure 3-4. Expression of microRNA biogenesis genes in cancer cachexia patients. (A) DROSHA, (B) DGCR8, (C) DICER1 and (D) AGO2. Data based on cachexia patient's microarray data and shown as mean ± SE.

3.3.4 Muscle wasting and growth pathways enriched with microRNA targets

MicroRNAs may function co-ordinately on molecular pathways. Pathway enrichment analysis can help identify microRNA targets enriched among known pathway genes. The KEGG pathway annotations were used to determine pathways enriched with miR-1/206 and miR-133a/b targets (Figure 3-5). The false discovery rate was calculated to limit identification of spurious pathways. Pathways significantly enriched with both miR-1/206 and miR-133a/b targets that may be regulated in cancer cachexia included the MAPK signaling pathway, the insulin signaling pathway, the JAK-STAT pathway, and regulation of actin cytoskeleton (Figure 3-5).

Gene ontology enrichment analysis was used to further explore the possible functional role of miR-1/206 and miR-133a/b down-regulation in pancreatic cancer cachexia patients. GO enrichment analysis was conducted to find GO molecular function terms, GO cellular component terms and GO biological process terms significantly enriched among miR-1/206 and miR-133a/b targets (Appendix 7-1). Molecular functions significantly enriched among miR-133a/b targets included protein phosphatase activity and transcription regulator activity (P <0.01, FDR <10%). Analysis of microRNA target expression found no evidence of miR-1, miR-133a, miR-133b or miR-206 action at the mRNA level in cachexia patients with varying weight-loss (Supplementary Figure 3-7), but this does not rule out miR-1, miR-133a, miR-133b or miR-206 action at the protein level in cachexia which was not measured in the present study.

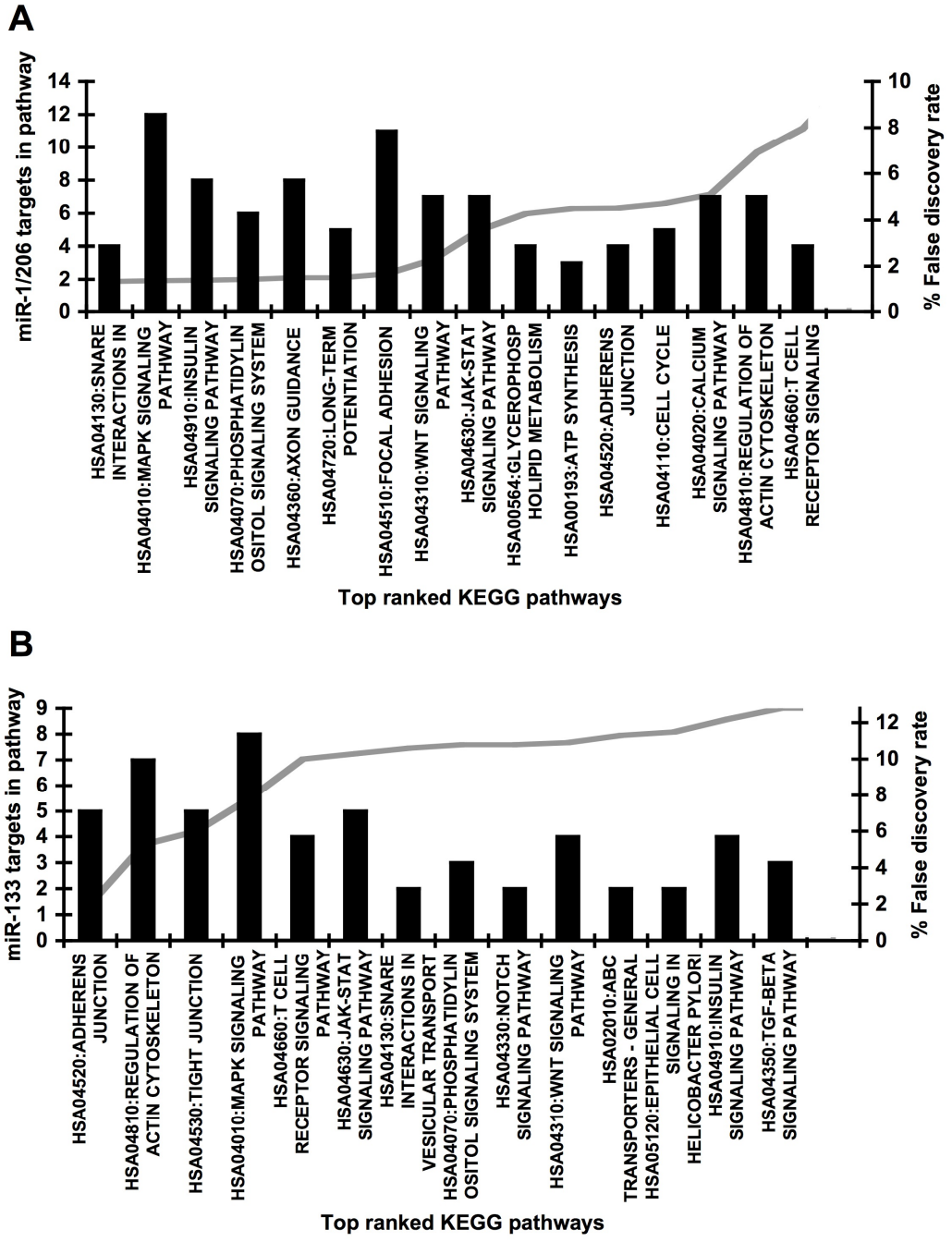


Figure 3-5. KEGG pathways enriched with (A) miR-1/206 and (B) miR-133 targets. Data shows number of microRNA targets (black-bars) and false discovery rate (grey-line) based on pathway enrichment analysis.

3.3.5 Primary and mature miR-21 expression in pancreatic cancer patients

Chronic inflammation and loss of muscle mass is a characteristic of ICU patients. However, it is not known whether the mechanism leading to loss of muscle mass in ICU patients also occurs in cancer cachexia patients. Recently published data from our laboratory showed mature miR-21 was unchanged in ICU patients, while Affymetrix probe data suggested primary miR-21 was significantly elevated (Fredriksson et al. 2008). RT-qPCR was used to confirm whether pri-miR-21 was elevated in the ICU patients. In the ICU patients, pri-miR-21 expression was significantly higher than controls ($P < 0.01$; Figure 3-6).

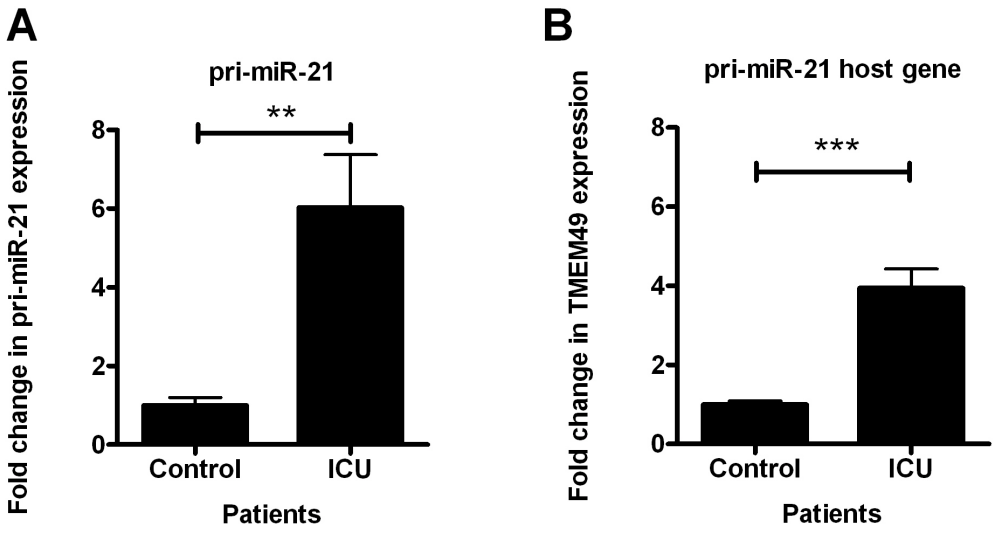


Figure 3-6. Fold change in (A) pri-miR-21 and (B) TMEM49 (host) gene expression in ICU patients with sepsis compared to control patients. Data shown as mean ± SE. *** $P < 0.001$ ** $P < 0.01$.

Furthermore, TMEM49 which is the host gene from which pri-miR-21 is transcribed was also significantly higher in the ICU patients (Figure 3-6) compared to the control patients ($P < 0.001$). The confirmation of large differences between pri-miR-21 and mature miR-21 expression in the skeletal muscle of ICU patients led to the hypothesis that pri-miR-21 or mature miR-21 could also be dysregulated in the skeletal muscle of cancer cachexia patients. Mature miR-21 expression was measured in pancreatic cancer patients classified into two groups either pre-cachexia with less than 10% weight loss (n = 10) or cachexia with more than 10% weight loss (n = 9) and compared to controls with no weight loss (n = 7). Mature miR-21 appeared to increase 50% in the pre-cachexia group and 120% in the cachexia group compared to controls (Figure 3-7).

However, the increase in mature miR-21 was not significant (F = 0.8, $P > 0.05$). There appeared to be a 90% increase in pri-miR-21 expression in the pre-cachexia compared to the control (Figure 3-7), but this was not significant (F = 1.1, $P > 0.05$).

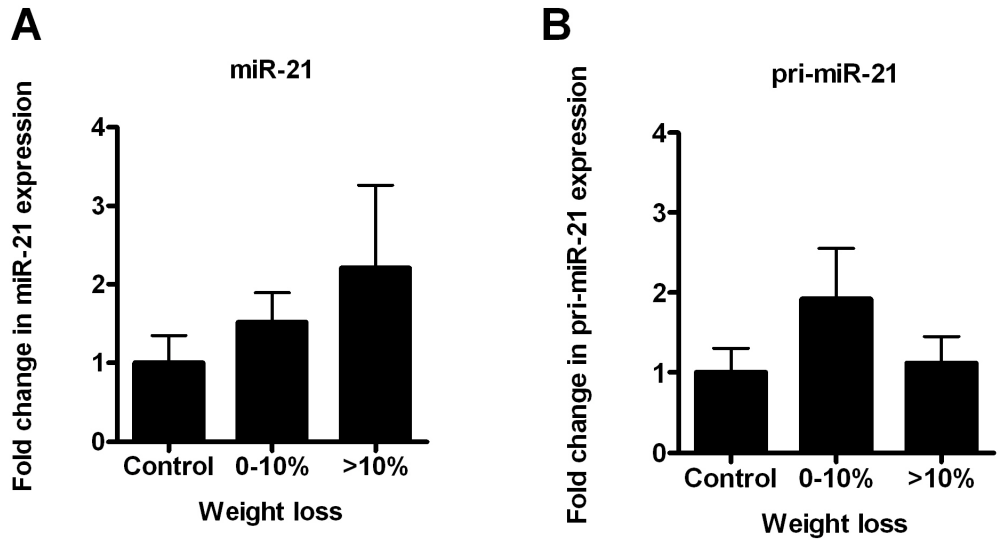


Figure 3-7. (A) Fold change in mature miR-21 and (B) pri-miR-21 expression in pancreatic cancer patients with pre-cachexia (0-10% weight loss) or cachexia (>10% weight loss) compared to controls. Data shown as mean ± SE.

3.3.6 Muscle wasting pathways enriched with miR-21 targets

Pathway enrichment analysis revealed miR-21 targets may be involved in several pathways, including JAK-STAT, MAPK signaling, apoptosis and cytokine-pathways (Figure 3-8). GO enrichment analysis of miR-21 targets associated with GO Molecular function, GO Cellular component and GO Biological process terms are summarised in Appendix 7-4. There was a significant enrichment of genes involved in ubiquitin-protein ligase activity among the miR-21 target genes ($P < 0.01$, FDR = 0%), including FBXO11, HIP2, RNF103, RNF111, TRIM33, UBE2D3, and UBE4A (Appendix 7-4).

There appeared to be no effect of miR-21 on target expression in cachexia patients at the mRNA level. No significant correlation was found between average miR-21 target expression and weight loss in patients (Supplementary Figure 3-9). This suggests any potential effect of miR-21 on muscle wasting pathways in cachexia occurs at the protein level, although target proteins were not measured in patients in the present study.

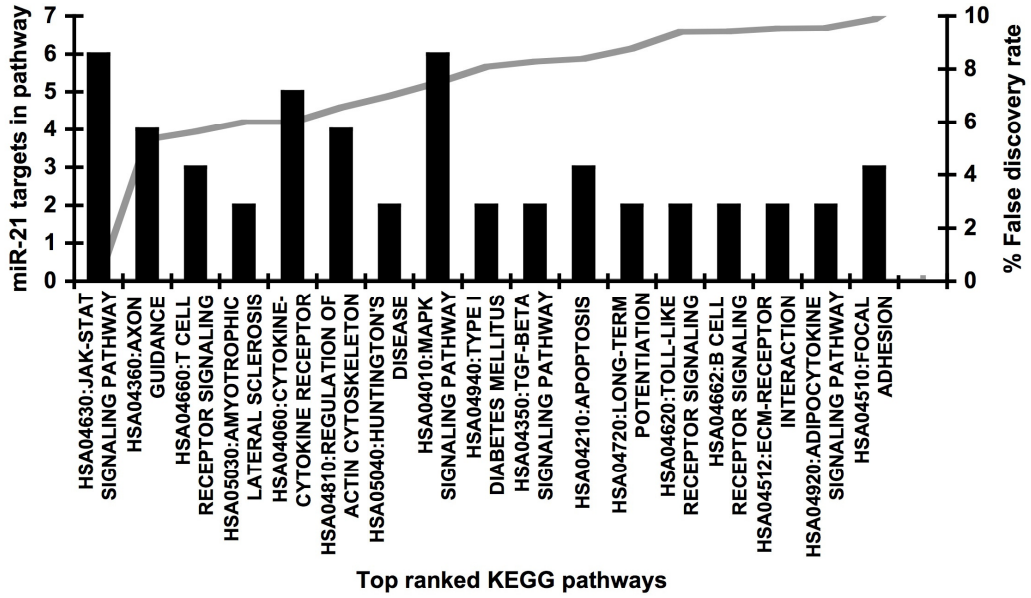


Figure 3-8. Top ranked KEGG pathways enriched with miR-21 targets. Data shows the number of miR-21 targets (black-bars) and false discovery rate (grey-line) based on pathway enrichment analysis.

3.3.7 Experimental validation of microRNA arrays

To search for further potential candidate microRNAs involved in cancer cachexia, microRNA arrays were conducted on pooled patient RNA (I. Gallagher, personal communication). Experimental validation was conducted using RT-qPCR based on a subset of cancer cachexia patients profiled on microarrays and microRNA arrays (n = 20; Supplementary Table 3-2) with varying tumour types.

The experimental validation was conducted on 7 microRNAs (Supplementary Table 3-3; miR-23a, miR-27b, miR-29a, miR-29b, miR-424, miR-195 and miR-143) identified as up-regulated and 1 microRNA (miR-208) identified as down-regulated during weight loss based on SAM analysis of microRNA array data.

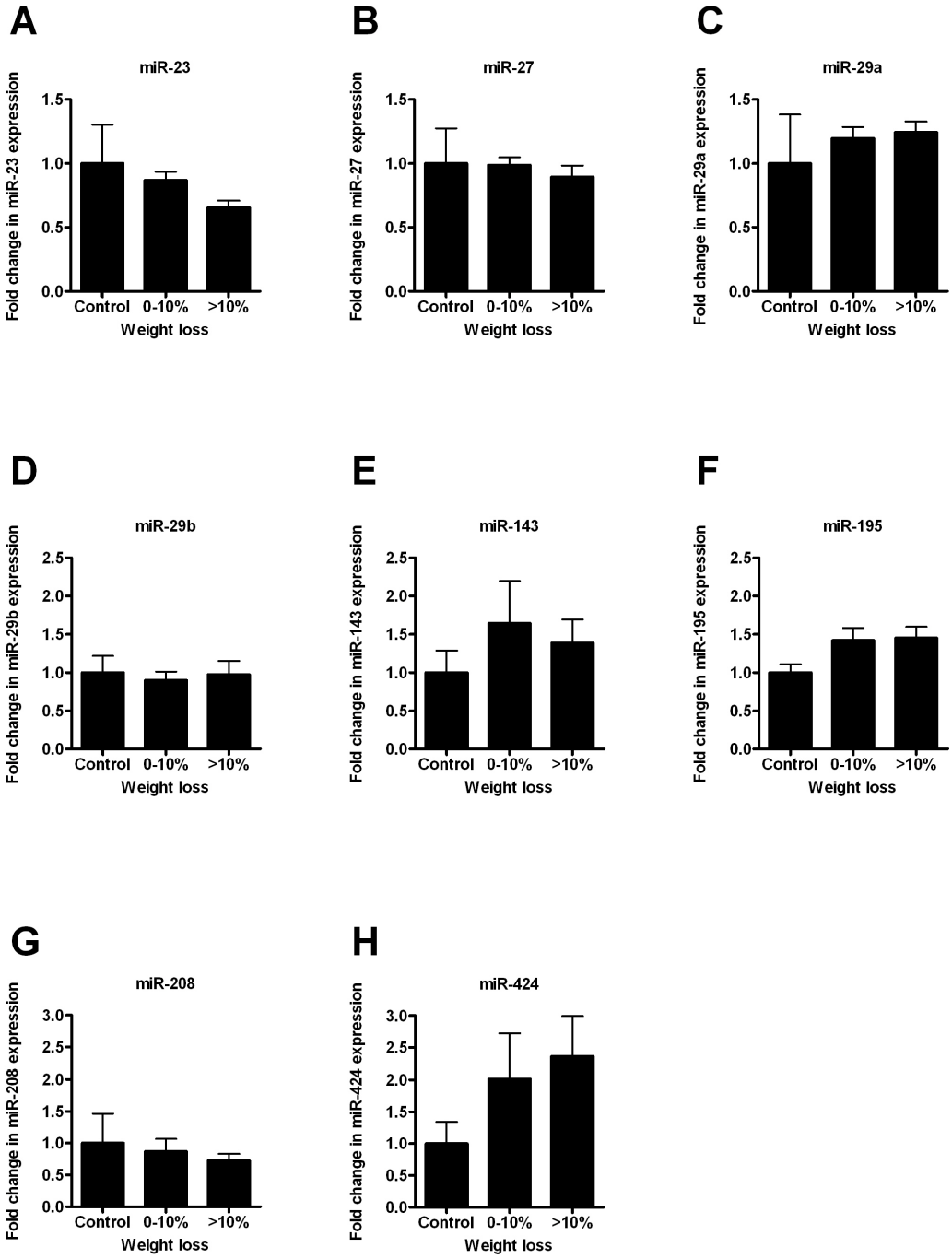


Figure 3-9. Fold change in (A) miR-23a, (B) miR-27b, (C) miR-29a, (D) miR-29b, (E) miR-143, (F) miR-195, (G) miR-208 and (H) miR-424 expression in skeletal muscle from cachexia patients with >10% weight loss, pre-cachexia patients with 0-10% weight loss and control patients (n = 20). Data normalised to control and presented as mean ± SE.

Suprisingly, there were no significant differences detected in miR-23a, miR-27b, miR-29a, miR-29b, miR-143, miR-195, miR-208 and miR-424 expression (Figure 3-9) between the cachexia, pre-cachexia and control group. The lack of any significant

dectectable differences may have been due to variation in microRNA expression within the patient groups ($F = 0.5$, $P > 0.05$).

3.3.8 Muscle wasting and growth pathways targeted by cachexia microRNAs

The present RT-qPCR results failed to confirm existing microRNA array data (I. Gallagher, personal communication). Nevertheless, pathway enrichment analysis of miR-143 and miR-195/424 targets revealed enrichment of pathways previously linked to cachexia including the ubiquitin-mediated proteolysis pathway and the apoptosis pathway (Supplementary Figure 3-8). Among the miR-195/424 targets were genes involved in the ubiquitin-mediated proteolysis pathway, including BTRC, CDC27, CUL2 and FBXW7. The miR-195/424 targets enriched among the apoptosis pathway genes included AKT3, BCL2, CAPN6, PIK3R1, PPP3CB, PRKACA and PRKAR2A. Interestingly, the muscle growth linked MAPK pathway was enriched with miR-23a, miR-27b, miR-143 and miR-195/424 targets (Figure 3-10; Supplementary Figure 3-8).

Pathway enrichment analysis of miR-23a targets revealed significant enrichment of genes involved in MAPK signaling, focal adhesion and ubiquitin-mediated proteolysis (Figure 3-10). The pathway enrichment analysis of miR-27b targets also revealed significant enrichment of genes associated with MAPK signaling and focal adhesion pathways. In addition, genes involved in insulin signaling and calcium signaling were enriched among miR-27b targets (Figure 3-10).

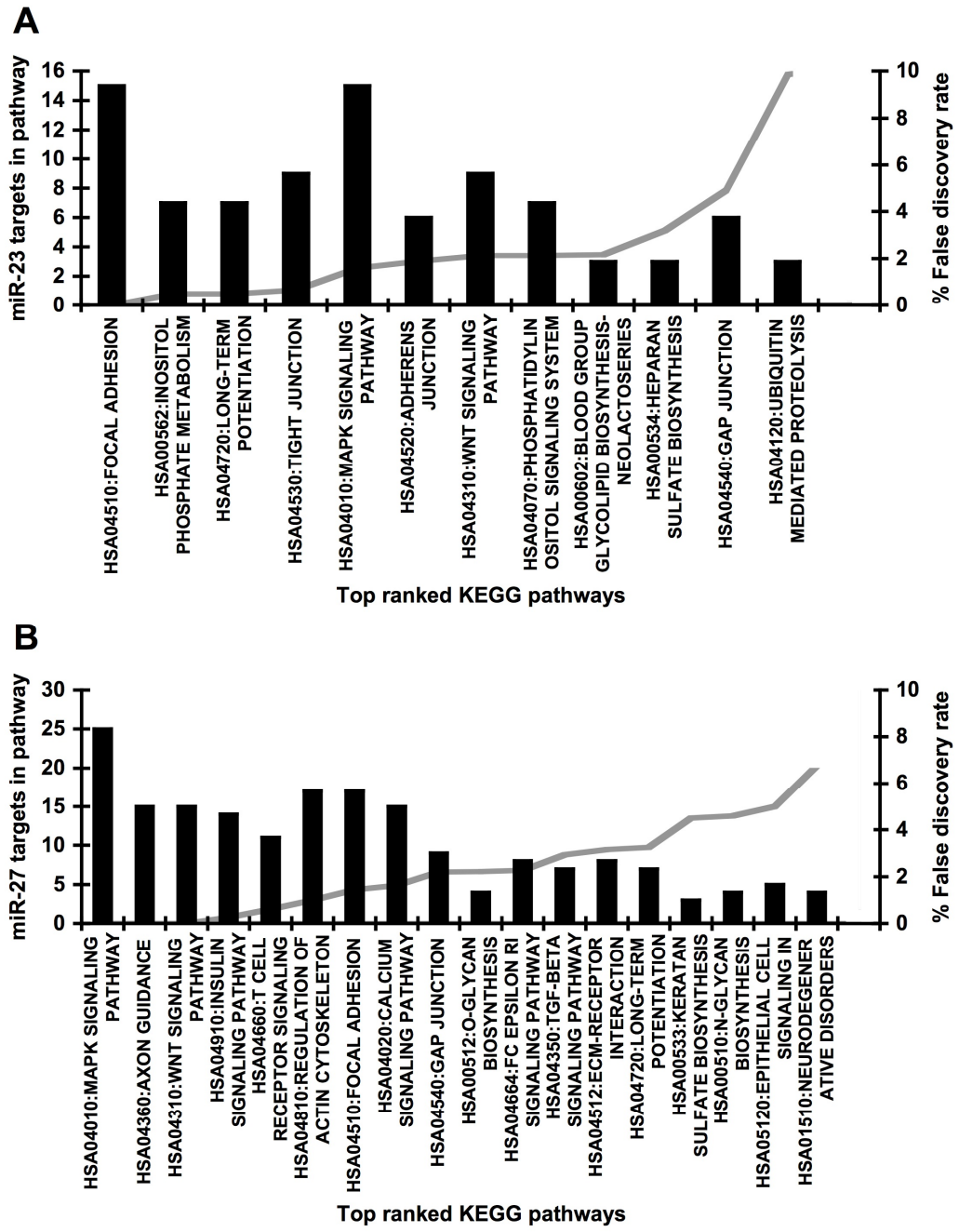


Figure 3-10. KEGG pathways enriched with (A) miR-23a and (B) miR-27b targets. Data shows number of microRNA targets (black-bars) and false discovery rate (grey-line) based on pathway enrichment analysis.

3.3.9 Cachexia microRNAs may target muscle wasting gene groups

GO enrichment analysis of GO Molecular function terms, GO Cellular component terms and GO Biological process terms associated with miR-23a, miR-27b, miR-143, miR-195/424 targets revealed enrichment of gene functions linked with muscle-wasting and muscle growth. Ubiquitin cycle genes were enriched among miR-27b targets (Appendix 7-10), which may contribute muscle protein breakdown via the ubiquitin mediated proteolysis pathway. Muscle myosin genes were found to be miR-23a targets, which is in agreement with the decreased myosin expression that has been reported in cachexia patients (Ramamoorthy et al. 2009; Schmitt et al. 2007). Cell proliferation and cell cycle genes were also enriched among miR-23a targets (Appendix 7-7). There was enrichment of miR-195/424 targets associated with ubiquitin dependent protein catabolism including BTRC, FBXO21, SIAH1, UBE4B, USP14, USP15, USP2, USP25, USP6 and USP9X (Appendix 7-22). Insulin-like growth factor genes and epidermal growth factor receptor genes were enriched among miR-143 targets including, ERBB3, ERBB4 and IGF1R, CTGF and IGFBF5 (Appendix 7-19).

3.3.10 Expression of miR-23a and miR-27b targets correlates with weight loss

MicroRNA target expression was examined to determine whether there was any *in-vivo* evidence of miR-23a, miR-27b, miR-29a, miR-29b, miR-424, miR-195, miR-143 or miR-208 action on weight loss in cancer cachexia patients. There were no correlations found between miR-29a, miR-29b, miR-424, miR-195, miR-143 or miR-208 target expression and weight loss (Supplementary Figure 3-9). However, there was a significant negative correlation between miR-23a and miR-27b target expression and patient weight loss (Figure 3-11; r = -0.56, P <0.01; r = -0.67, P <0.01).

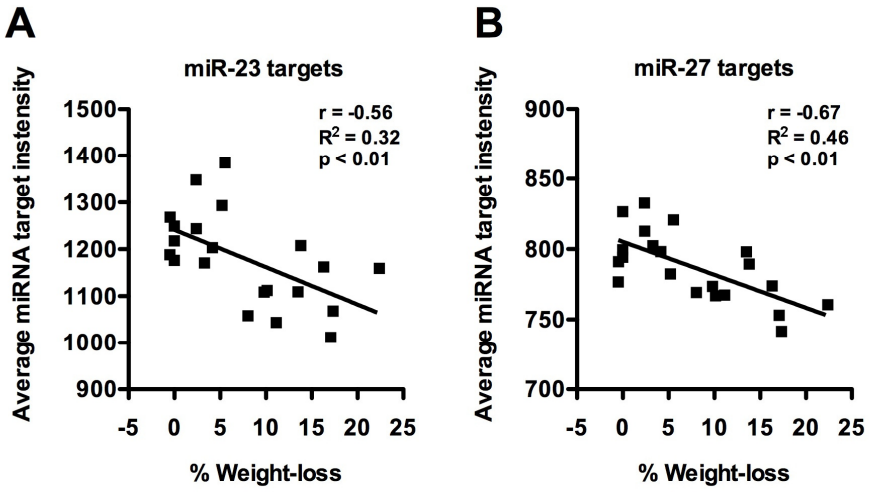


Figure 3-11. (A) miR-23a and (B) miR-27b targets correlate with weight loss. Average microRNA target intensity for each patient was determined from cachexia patient microarray data (n = 20).

Furthermore, there was a negative correlation between miR-143 target expression and patient weight loss (Supplementary Figure 3-9; r = -0.45, P <0.05). Interestingly, miR-23a is predicted to target MAFbx and MuRF1 (two specific muscle wasting associated genes). Evidence of *in-vivo* target suppression would be indicated by a negative correlation between target gene expression and microRNA expression (Bartel, 2009), but no correlation was found between miR-23a and MAFbx or MuRF1 expression.

3.4. Discussion

The pathogenesis of cancer cachexia is largely unknown and there are currently no early indicators of cachexia susceptibility among cancer patients (Tan & Fearon 2008; Bozzetti & Mariani, 2008; Skipworth et al. 2007; Tan et al. 2008). Yet it is well established that cachexia has major implications for quality of life, morbidity and mortality in cancer patients (Bruera, 1997). The focus of the present study was to identify potential biomarkers of cancer cachexia. MicroRNAs have emerged as novel biomarkers in several cancers and can be used to classify tumour types (Blenkiron et al. 2007; Lu et al. 2005; Szafranska et al. 2008), but whether microRNAs could be novel biomarkers of skeletal muscle wasting in cancer cachexia is unknown.

3.4.1 Evidence of muscle microRNAs as biomarkers of cancer cachexia

In the present study microRNAs known to be highly expressed in skeletal muscle and important for myogenesis were examined for association with cancer cachexia severity. Despite the significant weight loss in the cachexia group there were no significant differences in miR-1, miR-133a, miR-133b or miR-206 expression between cachexic patients and controls. The lack of any significant differences was somewhat surprising given the established role of these microRNAs in myogenesis and muscle growth (Kim et al. 2006; Nakajima et al. 2006; Chen et al. 2006; Boutz et al. 2007; van Rooij et al. 2008). There are other explanations that may explain the lack of differences in muscle growth associated microRNAs. For example, the heterogeneity in miR-1, miR-133a, miR-133b and miR-206 expression could be a reflection of the cachexia state. There has been no universal cause for cachexia identified, for some patients microRNA expression may be a factor but for others it may not be an important factor.

Alternatively, the classification of patients into pre-cachexia and cachexia groups perhaps hides the sensitivity of muscle microRNA expression to small changes in muscle mass. The idea of classifying cancer patients as pre-cachexic or cachexic based on below or above 10% weight loss was based on a proprosal by an international cancer cachexia working group (Bozzetti & Mariani, 2008). Instead, the association between individual patients' microRNA expression and weight loss was examined, which revealed a modest negative correlation between miR-133a expression and weight loss across cancer patients with different tumour types, but it was not possible to determine whether miR-133a expression was also affected by weight loss. The prevalence of

cachexia has been reported to be higher among pancreatic cancer patients compared to other cancer patients (Fearon, 1992). Therefore, miR-1, miR-133a, miR-133b and miR-206 expression was analysed again in the pancreatic cancer patients only. Interestingly, in the pancreatic cancer patients there was a significant association between miR-1, miR-133a, miR-133b and miR-206 expression and weight loss, which was not apparent when microRNA expression data from the entire cancer cachexia cohort (n = 58) was analysed. Although it appeared miR-133a was the strongest predictor of weight loss in the pancreatic cancer patients. Thus this study provides the first evidence that miR-1, miR-133a, miR-133b and miR-206 may be potential biomarkers of cancer cachexia and could be useful for identifying pancreatic cancer patients with early signs of skeletal muscle wasting.

3.4.2 Functional role of microRNAs in pancreatic cancer cachexia patients

Next it was important to establish whether these muscle microRNAs are not only potential biomarkers of cachexia, but also play a functional role in cachexia pathogenesis in pancreatic cancer patients. Gene ontology (GO) enrichment analysis was used to identify functional gene groups, which were targeted by miR-1, miR-133a, miR-133b and miR-206. Both miR-1 and miR-206 have many common targets as they are from the same microRNA family and have similar target seed sequences. Similarly, both miR-133a and miR-133b have many common targets, as they are from the same microRNA family and only differ by a single nucleotide. Biological processes enriched with over fifty miR-1/206 targets included regulation of cellular physiological process, regulation of cellular metabolism and regulation of transcription. Muscle development genes were also significantly enriched with miR-1/206 target sites. Molecular functions enriched among miR-1/206 targets included transcriptional regulator activity, which was also enriched among miR-133a/133b targets. The targeting of transcriptional regulators suggests miR-1/206 and miR-133a/133b may have widespread indirect effects on gene expression. The functional role of miR-1/206 and miR-133a/133b in cancer cachexia is not clear.

In C2C12 myoblasts miR-1 and miR-206 have both been shown to promote differentiation. The role of miR-133a in developing muscle is equivocal because miR-133a promotes proliferation in myoblasts (Chen et al. 2006), but it is strongly induced during myogenesis and differentiation of myoblasts into myotubes (Boutz et al. 2007; Rao et al. 2006). Conversely, this would suggest that down-regulation of miR-1, miR-

133a and miR-206 may be necessary for proliferation and stem-cell renewal. Previously, it has been suggested cachexia in skeletal muscle may lead to increases in muscle stem cells populations in an attempt to maintain the muscle mass (Berardi et al. 2008). The finding of elevated CD45 (+) hematopoietic stem cells in the skeletal muscle of C-26 tumour-bearing mice supports the idea that muscle regeneration may by occurring during cachexia (Berardi et al. 2008).

There are an increasing number of miR-1 (Yu et al. 2008; Vinther et al. 2006; Nakajima et al. 2006; Ikeda et al. 2009; Simon et al. 2008; Shan et al. 2009), miR-133a (Shan et al. 2009; Yin et al. 2008; Duisters et al. 2009; Chen et al. 2006; Boutz et al. 2007; Chen et al. 2009) and miR-206 (Anderson et al. 2006; Rosenberg et al. 2006; Kim et al. 2006; Boutz et al. 2007) targets which have been experimentally validated in different studies. Two studies have shown IGF-1 can be repressed by miR-1/206 in myoblasts (Yu et al. 2008; Shan et al. 2009). IGF-1 is a positive regulator of muscle growth and can inhibit FOXO1 mediated atrophy (Stitt et al. 2004). In multiple atrophy-inducing models IGF is down-regulated (Lecker et al. 2004). In colorectal cancer patients with cachexia IGF-1 is decreased, but in gastric cancer patients with cachexia IGF-1 is elevated (Huang et al. 2005). Therefore, the present data suggest IGF-1 targeting by miR-1/206 may be partly compensatory in cancer cachexia patients.

Global protein profiling using SILAC in HeLa cells transfected with a miR-1 mimic reported 11 proteins were suppressed and of these 6 were also repressed in a 3'UTR reporter assay (Vinther et al. 2006). These miR-1 targets included ADAR, HNRPU, DHX15, G6PD, CAP1 and TPM3. ADAR is an adenosine deaminase that acts on RNA converting adenosines to inosine known as A to I editing. In the human transcriptome widespread A to I editing has been reported (Athanasiadis et al. 2004), furthermore A to I editing in humans predominantly occurs in non-coding RNA regions. Therefore miR-1 targeting of ADAR could have potentially widespread effects, for example by modifying microRNA target sites in 3'UTRs. However, the function of ADAR in human skeletal muscle has not been examined. HNRPU is a heterogeneous nuclear ribonucleoprotein, which is involved in alternative splicing of genes (Castle et al. 2008) and has more recently been linked to microRNA processing (Guil & Cáceres, 2007; Michlewski et al. 2008). Both ADAR and HNRPU have not been previously linked to muscle wasting in cancer cachexia patients. MEF2A is a muscle transcription factor, reportedly negatively regulated by miR-1 (Ikeda et al. 2009; Simon et al. 2008),

although a MEF2 binding site may also regulate transcription of primary transcripts encoding miR-1, miR-133 and miR-206 (Liu et al. 2007). Muscle wasting in cancer cachexia patients has not been previously associated with MEF2A.

Targets of miR-133a have been experimentally validated in a variety of cell types, but it is unknown how relevant these targets are in skeletal muscle. Overexpression of miR-133 in cardiomyocytes decreased CTGF protein and production of collagens (Duisters et al. 2009). Furthermore, miR-133 was shown to target TGIF-β in fibroblasts and overexpression of miR-133 increased cellular collagen (Duisters et al. 2009). TGIF-β treatment in nude mice is reported to be associated with interstitial fibrosis and cachexia (Zugmaier et al. 1991), but there have been no reports yet of fibrosis occurring in cancer cachexia patients. In addition, TGF-β inhibits myogenesis mediated by CDC42 and activation of the JNK pathway (Meriane et al. 2002). These studies suggest miR-133 down-regulation may promote fibrosis.

Taken together the evidence from studies experimentally validating miR-1, miR-133 and miR-206 targets suggest their down-regulation in pancreatic cancer patients with cachexia may trigger positive and negative responses in skeletal muscle. Targeting of muscle transcription factors and translational regulators such as HNRNP suggests miR-1, miR-133 and miR-206 may have widespread indirect effects. However, a global proteomic profiling approach would be required to establish the importance and relevance of miR-1, miR-133 and miR-206 regulation in skeletal muscle during cancer cachexia pathogenesis *in-vivo*.

3.4.3 Evidence of miR-21 as a biomarker of cancer cachexia

MicroRNA-21 is reported to be up-regulated in cancer (Iorio et al. 2005). In the present study miR-21 expression was measured in cancer cachexia patients to determine whether it was a biomarker of cancer cachexia severity. Increases in pri- and mature miR-21 expression were observed in cachexia patients with >10% weight loss, but the >100% change was not significant. The pathway enrichment and GO enrichment analysis suggested miR-21 might have a functional role in cancer cachexia. Targets of miR-21 were enriched in the JAK-STAT, MAPK signaling, apoptosis and cytokine-cytokine pathways. Furthermore, miR-21 targets were enriched for ubiquitin-protein ligase activity and increased ubiquitin-proteosome components have been reported in cancer patients. For example, significantly higher ubiquitin and 20S proteasome

subunit mRNA expression was found in skeletal muscle from cancer patients (Williams et al. 1999). In addition, ubiquitin mRNA was found to be higher in gastric cancer patients than control patients (Bossola et al. 2003). However, the increase in miR-21 in cancer cachexia patients would suggest miR-21 may be a post-transcriptional activator of ubiquitin-protein ligase targets which would be unusual as most microRNAs suppress target expression (Bartel, 2009). On the other hand miR-21 could be up-regulated in cancer cachexia patients as a compensatory mechanism to reduce ubiquitin-system activity

Past studies have shown apoptosis genes are targeted by miR-21 in cancer cells. For example, knockdown of miR-21 activates caspases and leads to increased apoptotic cell death in glioblastoma cells (Chan et al. 2005). The pro-inflammatory cytokine IL-6 has been shown to induce miR-21 expression in myeloma cells mediated by STAT3 (Löffler et al. 2007). Pro-inflammatory cytokines including IL-6 have been associated with survival in pancreatic cancer cachexia patients (Moses et al. 2009). These studies suggest IL-6 could be an inducer of miR-21 in cancer cachexia patients. Recently miR-21 was shown to target SPRY1 augmenting the MAPK/ERK pathway in fibroblasts contributing to myocardial disease (Thum et al. 2008b), which supports the present pathway enrichment analysis showing miR-21 targets were significantly enriched among the MAPK pathway genes. Furthermore, activation of MAPK/ERK in skeletal muscle has been reported in response to skeletal muscle overload such as resistance exercise and promotes skeletal muscle growth (Carlson et al. 2001).

Taken together these studies suggest miR-21 is a negative regulator of cell survival triggering apoptosis and ubiquitin-protein ligase activity, which is counterintuitive as miR-21 was up-regulated in cancer cachexia patients in the present study. However, miR-21 up-regulation in cachexia may also suppress MAPK pathway genes associated with muscle growth. Importantly, there have been no studies to date validating miR-21 targets in skeletal muscle. Therefore, the function of miR-21 in skeletal muscle remains largely unknown.

3.4.4 Experimental validation of microRNA arrays

To identify further potential microRNA biomarkers of cancer cachexia, RT-qPCR was conducted to measure microRNAs indicated as differentially expressed in cachexia based on microRNA array data (I. Gallagher, personal communication). These were

miR-23a, miR-27b, miR-29a, miR-29b, miR-143, miR-195, miR-424 indicated as up-regulated and miR-208 indicated as down-regulated in cachexia by the microRNA arrays (I. Gallagher). However, the RT-qPCR microRNA expression data were equivocal, as the significant differential expression of these microRNAs shown by microRNA arrays was not observed in the present study when individual patient microRNA expression was measured using RT-qPCR. There appeared to be large variability in the RT-qPCR microRNA expression data, thus differences between control, pre-cachexia and cachexia groups were not found to be significant. There appeared to be between 30-100% changes in miR-29a, miR-143, miR-195 and miR-424, while miR-23a, miR-27b and miR-208 appeared to be reduced by 10-35% in cachexia patients.

A possible explanation for the discrepancy between microRNA array data and RT-qPCR microRNA expression data may be that patient RNA was pooled for the microRNA arrays to reduce costs, while microRNA expression was measured using RT-qPCR in individual patients. Pooling patient RNA may have reduced the effects of biological variance during the microRNA array analysis, while true biological variance in cachexia patients was retained in the analysis of microRNA RT-qPCR expression data. Therefore it would be advisable when attempting to identify novel microRNA biomarkers of chronic diseases using microRNA array profiling to use individual patient RNA if costs and time allow.

Intriguingly, the pathway enrichment analysis indicated miR-195/424 target genes BTRC, CDC27, CUL2 and FBXW7 are involved in ubiquitin-mediated proteolysis. In addition, apoptosis pathway genes were enriched among miR-195/424 targets. The results of the gene ontology enrichment analysis further suggested miR-195/424 involvement in protein degradation, as miR-195/424 targets were found to be associated with ubiquitin dependent protein catabolism and ubiquitin cycle functions. Conversely, positive regulators of muscle anabolism and growth were found enriched among miR-143 targets, including insulin-like growth factor genes and epidermal growth factor receptor genes. The GO and pathway enrichment analysis also identified ubiquitin proteolysis genes as predicted targets of miR-23a and miR-27b, therefore the expression of miR-23a and miR-27b targets was examined further.

3.4.5 Evidence of miR-23a and miR-27b action on targets in cancer cachexia

Global analysis of microRNA target expression using microarray data can provide evidence of microRNA function via mRNA cleavage (Lim et al. 2005; Sood et al. 2006; Arora & Simpson, 2008). Past studies have reported suppression of hundreds of miR-133 target mRNAs in response to miR-133 overexpression in HeLa cells (Lim et al. 2005). In addition, microRNA expression levels have been shown to inversely correlate with mRNA target expression in different tissues (Lim et al. 2005; Sood et al. 2006; Arora & Simpson 2008). In skeletal muscle inverse correlations are reported between up-regulated microRNAs and down-regulated target mRNAs (Arora & Simpson, 2008). It has been suggested that existing patient microarray data can be mined for evidence of microRNA regulation (Arora & Simpson, 2008). Microarray data was available from the cancer cachexia patients in the present study. A microRNA target expression signature was determined for each microRNA measured based on averaging the expression of all mRNAs with predicted microRNA binding sites for each patient. Linear regression was used to determine whether the patient microRNA target signatures were associated with cancer cachexia severity. The miR-23a and miR-27b target signatures were negatively associated with weight loss, which suggests miR-23a and miR-27b targets may be suppressed in cachexia patients with high weight loss in accordance with microRNA array expression data (I. Gallagher, personal communication).

Skeletal muscle atrophy has been consistently linked with increased MAFbx and MuRF1 expression (Lecker et al. 2004). Interestingly, both MAFbx and MuRF1 harbour a miR-23a binding site in their 3'UTRs. However, there was no significant association between miR-23a expression and MAFbx or MuRF1. This does not preclude the possibility that miR-23a may target MAFbx or MuRF1 for translation repression without target mRNA degradation.

3.4.6 MicroRNA processing genes are unchanged in cancer cachexia

Changes in microRNA processing genes may help explain the down-regulation of miR-1, miR-133a, miR-133b and miR-206 observed here in skeletal muscle from pancreatic cancer patients with higher weight loss. The main microRNA processing genes that have been studied previously are DROSHA, DICER and AGO2 (Murphy et al. 2008). In the present study no differences in the expression of the main microRNA processing genes were found between control, pre-cachexia and cachexia patients, but this does not

preclude that post-transcriptional changes in the expression of microRNA processing genes occurred in the cachexia patients. In ovarian cancer DICER and DROSHA mRNA was reported to be down-regulated and higher DROSHA and DICER levels were associated with increased patient survival (Merritt et al. 2008). In contrast, in metastatic prostrate adenocarcinoma DROSHA was shown to be up-regulated with other microRNA processing genes (Ambs et al. 2008) suggesting that in the spread of prostate cancer DROSHA up-regulation may be important. Together these studies suggest microRNA machinery may play a positive or a negative role in different cancers.

In skeletal muscle DROSHA and EXPORTIN5 have been shown to increase during hypertrophy (McCarthy & Esser, 2007). However, microRNA processing genes were reported to be unchanged during muscle atrophy (McCarthy et al. 2007). Evidence from DICER mutant mouse embryonic stem cells suggests muscle stem cells lacking DICER would have severe defects in differentiation (Kanellopoulou et al. 2005). Intriguingly, DICER protein levels are inhibited by multiple cellular stresses such as reactive oxygen species and interferons, (Wiesen & Tomasi, 2009).

Cancer cachexia pathogenesis is linked with elevation of pro-inflammatory cytokines, which may activate cellular stress pathways in skeletal muscle (Seruga et al. 2008; Stephens et al. 2008; Busquets et al. 2007; Moses et al. 2009). Therefore, microRNA processing genes in skeletal muscle may be influenced by pro-inflammatory cytokines in cancer cachexia patients. However, in the present study there was no evidence of alteration of DICER gene expression in cancer cachexia patients. This may be because changes in DICER gene expression in response to cellular stress can occur post-transcriptionally (Wiesen & Tomasi, 2009), so would not be evident from gene expression data.

An alternative explanation is that down-regulated mature miR-1, miR-133a, miR-133b and miR-206 expression could be due to decreased primary miR-1, miR-133a, miR-133b and miR-206 transcription. MyoD binding sites have been identified on the primary transcripts encoding miR-1, miR-133a and miR-206 (Liu et al. 2007; Rao et al. 2006; Kim et al. 2006). In addition, MyoD protein has been reported to be decreased in gastro-intestinal cancer patients with cachexia (Busquets et al. 2007). However, primary microRNA transcription was not measured in the cancer cachexia patients in the present study.

3.4.7 Limitations in using weight loss as an indicator of cachexia severity

Weight loss is used as a universal indicator of cancer cachexia severity in clinical studies (Fearon, 1992). In the present study, the severity of cachexia in patients was based on weight loss calculated from body mass during clinical examination and patient estimates of body mass prior to cancer diagnosis. Therefore, the reliability of patient reported body mass may have influenced the grouping of patients as pre-cachexia or cachexia. Furthermore, defining cachexia severity based on weight loss assumes fat or muscle loss has the same functional consequences, but loss of muscle can lead to decreased muscle strength and potentially quality of life (Weber et al. 2009).

In addition, it was not considered whether patients' initial body mass could have influenced patient classification into pre-cachexia or cachexia groups. For example, a patient with an initial BMI of 40 before cancer, but during clinical examination has a BMI of 30 would be classified with significant cachexia. However, the large reduction in BMI may be mostly due to loss of fat mass, therefore the functional consequences would be minimal. In contrast, a patient with an initial BMI of 25 before cancer diagnosis but during clinical examination has a BMI of 20, may be classified as pre-cachexia, despite most likely greater loss of muscle mass.

In the current study it was not possible to measure change in muscle mass directly. Alternative body composition techniques, which could be used in future include under-water weighing, air displacement plethsmography, DEXA or MRI (Fouladiun et al. 2005; Woodrow, 2009). These body composition techniques all provide an estimate of muscle and fat mass, thus would provide a better indication of cachexia severity.

3.4.8 Limitations in the approach to identify microRNA biomarkers

A cross-sectional study to identify microRNA biomarkers only indicates microRNA expression in patients at the time of clinical examination and assumes patient variability in microRNA expression is consistently associated with cachexia severity. The variability in microRNA expression in the control patients may have contributed to the lack of statistical significance despite the 50-100% changes in microRNA expression. Therefore a longitudinal design would have been better to determine whether microRNAs are sensitive to the early changes in skeletal muscle occurring in the pre-cachexia state

Other factors that may influence the variability in microRNA expression in skeletal muscle independent of cachexia severity include ageing (Drummond et al. 2008) and cancer type. The cachexia patient groups were older than the control group and age is reported to affect skeletal muscle microRNA expression (Drummond et al. 2008). The initial analysis of microRNA expression in the entire cachexia cohort (n = 58) included patients with different cancer types and suggested miR-1, miR-133a, miR-133b and miR-206 were not biomarkers of cachexia. However, further analysis revealed miR-1, miR-133a, miR-133b and miR-206 expression in pancreatic cancer patients was significantly associated with cachexia severity. Therefore, the present findings suggest the importance of microRNAs as biomarkers of cachexia may not be universal across different cancer types, but may be limited to pancreatic cancer patients, although this would need to be examined in a larger patient cohort of different cancer types.

3.4.9 Limitations in isolation of RNA and determination of RNA quality.

RNA was isolated from over 120 muscle biopsies from cancer cachexia patients as part of this study, although microRNA expression was measured in only 58 patients due to poor RNA quality or insufficient RNA. All RNA was analysed in the same laboratory using an identical protocol, but there was degradation evident in some samples.

It is possible RNA degradation occurred prior to isolation, as there may have been a time lapse between performing the muscle biopsy in theatre and storing the muscle biopsy at -80°C. In addition, some patient muscle biopsy samples may have been defrosted due to a fault in the -80°C freezer at the Royal Infirmary, Edinburgh. Six months after initial RNA isolation and RNA quality determination, RNA was analysed using a Nanodrop spectrophotometer for evidence of RNA contamination.

There was a presence of RNA contamination in some patient samples, which may have influenced the efficiency of RT-qPCR for the determination of microRNA expression, thus all patient samples were reprecipitated in 100% ethanol. Despite the loss of patient samples due to RNA degradation, miR-1, miR-133a, miR-133b and miR-206 expression was measured in 58 patients, which should have been sufficient to detect whether there were significant differences between control, pre-cachexia, and cachexia groups or whether microRNAs may be biomarkers of cachexia severity.

The pathway enrichment and gene ontology enrichment analysis was useful to provide an overview of possible microRNA functions in cancer cachexia. However, it is based on computational predictions of microRNA targets. Therefore, false positive predictions may influence both the pathway and gene ontology enrichment analysis. Gene ontology enrichment analysis can be difficult to interpret due to the enrichment of large gene groups associated with a global cellular function such as metabolism or binding. Despite this the enrichment analysis did highlight pathways and gene ontologies associated with cancer cachexia.

3.4.10 Future research directions

MicroRNAs appear to be biomarkers of cancer cachexia severity in pancreatic cancer patients. Therefore, microRNAs may facilitate earlier diagnosis of cachexia before substantial muscle loss and decrease in quality of life. Longitudinal studies are needed to determine whether microRNAs are sensitive to the early changes in skeletal muscle occurring in the pre-cachexia state compared to other inflammatory biomarkers such as CRP and TNFα. It would be advantageous in future studies to use more sensitive indicators of cancer cachexia severity, for example muscle-mass loss determined by MRI or DEXA scans. In addition, it would be useful to examine serum microRNA levels, to determine whether serum microRNA levels reflect changes in skeletal muscle microRNAs, as muscle biopsies are not ideal for routine clinical testing.

Bioinformatics approaches such as pathway and gene ontology enrichment analysis are useful for examining microRNA function in disease. But further work to refine existing microRNA target prediction algorithms to be context specific and experimentally validate targets would facilitate interpretation of pathway and gene-ontology enrichment analysis. From the perspective of microRNA function in cachexia, new approaches to measure global changes in microRNA targets in patient skeletal muscle would provide more robust evidence of the magnitude of microRNA regulation in cancer cachexia pathogenesis.

3.4.11 Conclusions

- Potential microRNA biomarkers of cancer cachexia severity were identified in pancreatic cancer patients. These included miR-1, miR-133a, miR-133b and miR-206, which were inversely associated with weight loss and cancer cachexia severity.
- Experimental validation or microRNA arrays in cancer cachexia patients revealed further microRNAs including miR-23a and miR-27b which may be involved in the cancer cachexia pathogenesis, but which would require further validation in a larger patient cohort.
- MAFbx and MuRF1, two E3 ligases associated with muscle wasting, are predicted targets of miR-23a. However, there was no evidence of the affect of miR-23a on MAFbx or MuRF1 cleavage in patient skeletal muscle
- Further longitudinal studies are required to determine whether microRNAs play a functional role in cancer cachexia pathogenesis or are mainly biomarkers of the disease process.

3.5. Supplementary Data

Supplementary Table 3-1. Individual patient characteristics for all cancer cachexia patients used in miR-1, miR-133a, miR-133b and miR-206 analysis (n = 58).

Code	Classification	Age	Sex	Tumour Site	BMI	% weight loss
F113	Control	67	M	None	31.5	0.0
F175	Control	58	M	None	35.9	0.0
F176	Control	28	F	None	32.8	0.0
F30	Control	69	M	None	28.5	0.0
F119	Control	46	F	None	31.1	0.0
F153	Control	48	M	None	25.2	0.0
F33	Control	41	M	None	29.3	0.0
F111	Pre-cachexia	75	M	Pancreas	26.1	3.4
F114	Pre-cachexia	52	M	Oesophagus	25.5	4.1
F122	Pre-cachexia	58	M	OGJ	26.3	-0.5
F132	Pre-cachexia	66	M	Oesophagus	30.4	2.4
F124	Pre-cachexia	65	M	Pancreas	28.1	3.2
F126	Pre-cachexia	65	M	Gastric	29.4	0.0
F28	Pre-cachexia	64	M	Gastric	28.1	-0.5
F134	Pre-cachexia	76	M	Gastric	24.1	2.4
F141	Pre-cachexia	65	F	OGJ	31.3	4.9
F144	Pre-cachexia	76	F	Gastric	30.7	2.0
F150	Pre-cachexia	75	F	Gastric	22.9	3.7
F40	Pre-cachexia	70	M	Oesophagus	26.1	3.3
F167	Pre-cachexia	74	F	Duodenum	21.6	5.0
F169	Pre-cachexia	60	F	Pancreas/ duodenum	29.6	0.6
F171	Pre-cachexia	66	M	Pancreas, Gallbladder	29.7	1.7
F173	Pre-cachexia	83	F	Pancreas		4.2
F182	Pre-cachexia	70	M	OGJ	26.8	0.7
F183	Pre-cachexia	69	M	Gastric	26.1	0.0
F44	Pre-cachexia	83	M	Gastric	24.6	2.4
F32	Pre-cachexia	70	M	Oesophagus	25.9	2.5
F120	Pre-cachexia	79	M	CBD	25.1	5.5
F143	Pre-cachexia	76	F	Pancreas	26.9	9.8
F152	Pre-cachexia	62	F	Pancreas	25.2	8.0
F29	Pre-cachexia	49	M	Oesophagus	28.2	5.2
F136	Pre-cachexia	75	M	Oesophagus	24.6	7.1
F138	Pre-cachexia	70	M	Gastric	24.7	5.7
F155	Pre-cachexia	52	M	Pancreas	27.1	7.7
F163	Pre-cachexia	52	M	Pancreas	30.3	6.1
F168	Pre-cachexia	45	M	OGJ	21.4	7.5
F172	Pre-cachexia	69	M	Gastric	26.4	6.4
F181	Pre-cachexia	65	M	Pancreas	24.4	9.5
F38	Pre-cachexia	69	M	Gastric	27.6	7.6
F129	Cachexia	72	M	Pancreas	24.1	13.8

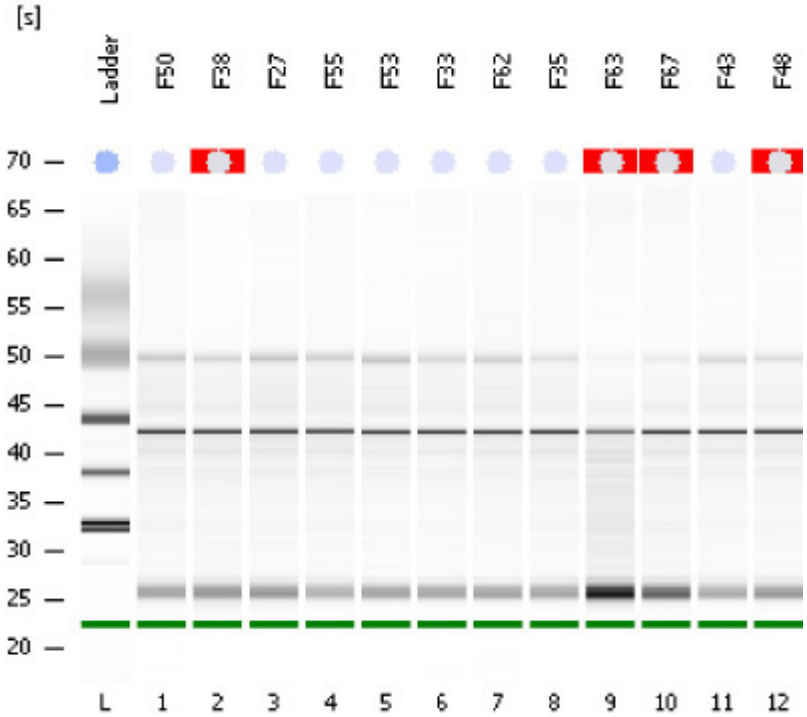
F140	Cachexia	75	F	Pancreas	20.1	11.1
F46	Cachexia	63	F	Pancreas	23.2	13.5
F116	Cachexia	50	M	OGJ	26.7	13.2
F145	Cachexia	62	F	Gastric	25.2	11.6
F146	Cachexia	73	M	Gastric	21.0	12.8
F151	Cachexia	55	F	Pancreas	20.5	12.8
F159	Cachexia	62	F	Oesophagus	23.1	14.5
F164	Cachexia	65	M	OGJ	26.2	11.7
F165	Cachexia	54	M	OGJ	32.0	14.7
F27	Cachexia	67	M	Gastric	34.4	12.9
F118	Cachexia	61	M	Oesophagus	24.3	16.3
F121	Cachexia	69	F	Pancreas	20.5	17.3
F156	Cachexia	71	M	Small bowel	21.1	17.1
F148	Cachexia	71	F	Pancreas	16.7	16.5
F162	Cachexia	65	F	Gastric	27.1	16.1
F166	Cachexia	75	F	Pancreas	21.4	16.2
F157	Cachexia	66	F	Pancreas	19.1	22.4
F158	Cachexia	44	F	Pancreas	21.7	43.8

Supplementary Table 3-2. Characteristics of patients used for experimental validation of microRNA arrays, microRNA processing gene expression analysis and microRNA target expression analysis (n = 20).

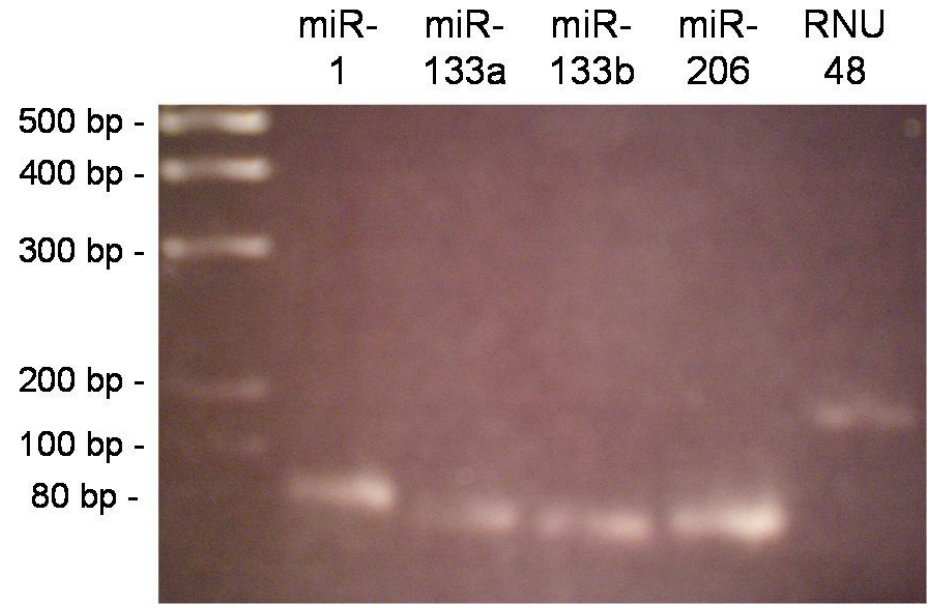
Code	Classification	Age	Sex	Tumour Site	BMI	% weight loss
F129	Cachexia	72	M	Pancreas	24	13.8
F140	Cachexia	75	F	Pancreas	20	11.1
F46	Cachexia	63	F	Pancreas	23	13.5
F118	Cachexia	61	M	Oesophagus	24	16.3
F121	Cachexia	69	F	Pancreas	21	17.3
F156	Cachexia	71	M	Small bowel	21	17.1
F157	Cachexia	66	F	Pancreas	19	22.4
F114	Pre-cachexia	52	M	Oesophagus	26	4.1
F122	Pre-cachexia	58	M	OGJ	26	-0.5
F132	Pre-cachexia	66	M	Oesophagus	30	2.4
F28	Pre-cachexia	64	M	Gastric	28	-0.5
F40	Pre-cachexia	70	M	Oesophagus	26	3.3
F44	Pre-cachexia	83	M	Gastric	25	2.4
F120	Pre-cachexia	79	M	CBD	25	5.5
F143	Pre-cachexia	76	F	Pancreas	27	9.8
F152	Pre-cachexia	62	F	Pancreas	25	8.0
F29	Pre-cachexia	49	M	Oesophagus	28	5.2
F119	Control	46	F	HC	31	0.0
F153	Control	48	M	HC	25	0.0
F33	Control	41	M	HC	29	0.0

Supplementary Table 3-3. Fold change in microRNA probe expression between cachexia patients with high weight-loss and controls from microRNA arrays (I. Gallagher, personal communication). Experimental validation was performed on these microRNAs using RT-qPCR and data is shown in 3.3.7.

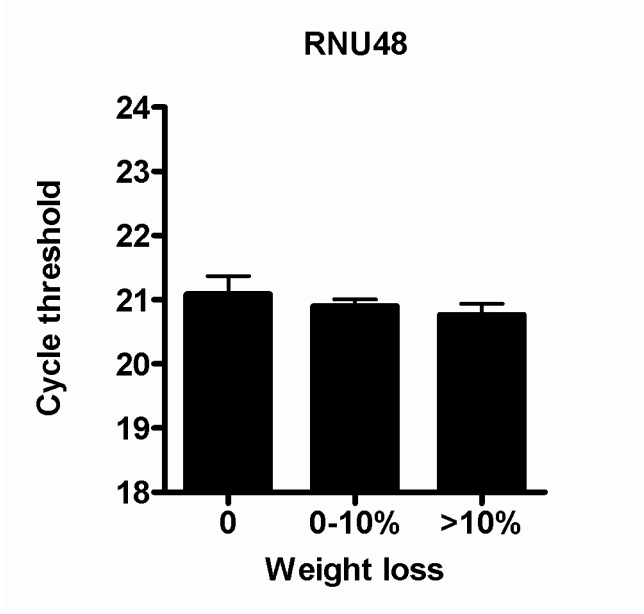
microRNA	Fold Change
hsa-miR-143/mmu-miR-143/rno-miR-143	1.98
hsa-miR-195/mmu-miR-195/rno-miR-195	1.22
hsa-miR-23a/mmu-miR-23a/rno-miR-23a	1.32
hsa-miR-27b/mmu-miR-27b/rno-miR-27b	1.42
hsa-miR-29a/mmu-miR-29a/rno-miR-29a	1.30
hsa-miR-29b/mmu-miR-29b/rno-miR-29b	1.69
hsa-miR-424	1.51



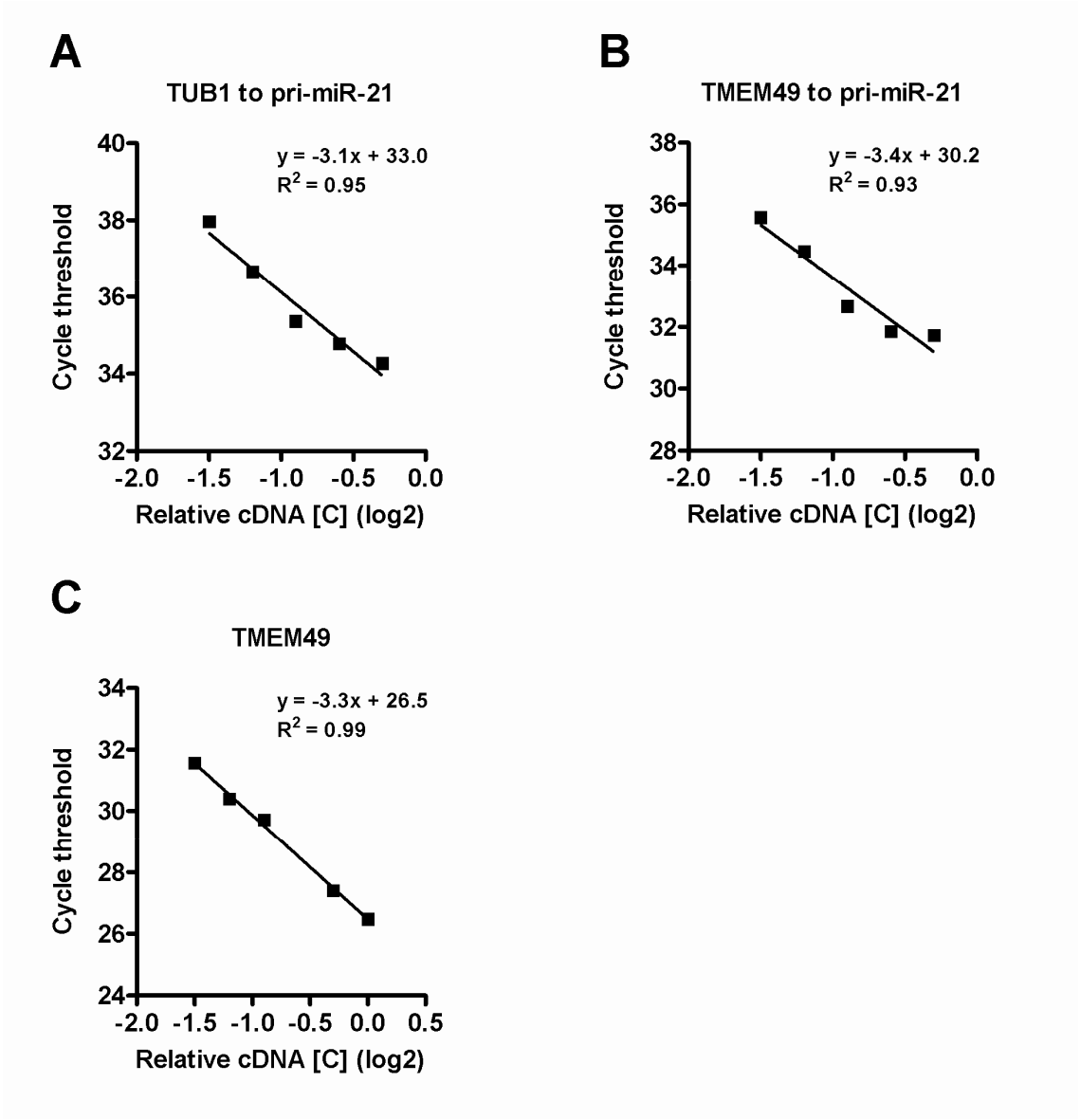
Supplementary Figure 3-1. Example of micro-electrophoresis gel showing RNA quality from cancer cachexia and control patient RNA samples during early stages of RNA isolation for the entire cachexia cohort. Clear evidence of RNA degradation in lane 9-10, with small RNA fragments detected at bottom of electrophoresis gels. RIN scores indicating RNA quality were below normal in lanes 2, 9-10 and 12.



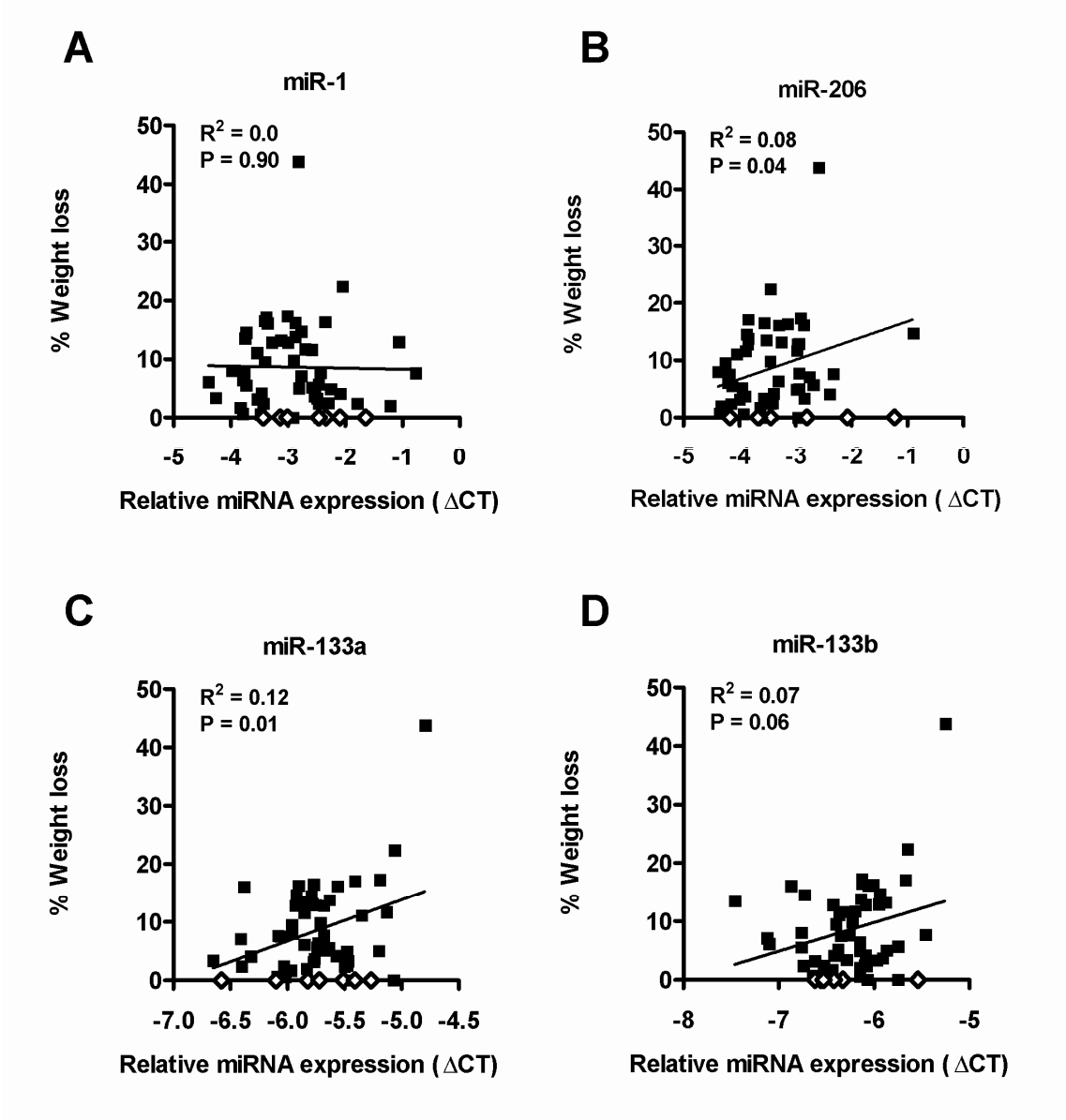
Supplementary Figure 3-2. Agarose gel showing specificity of microRNA Taqman assays to detect mature microRNAs. Mature miR-1, miR-133a, miR-133b, miR-206 are dectected around 80 bp as during reverse transcription, 60 nt stem-loop primers anneal to mature microRNA sequences.



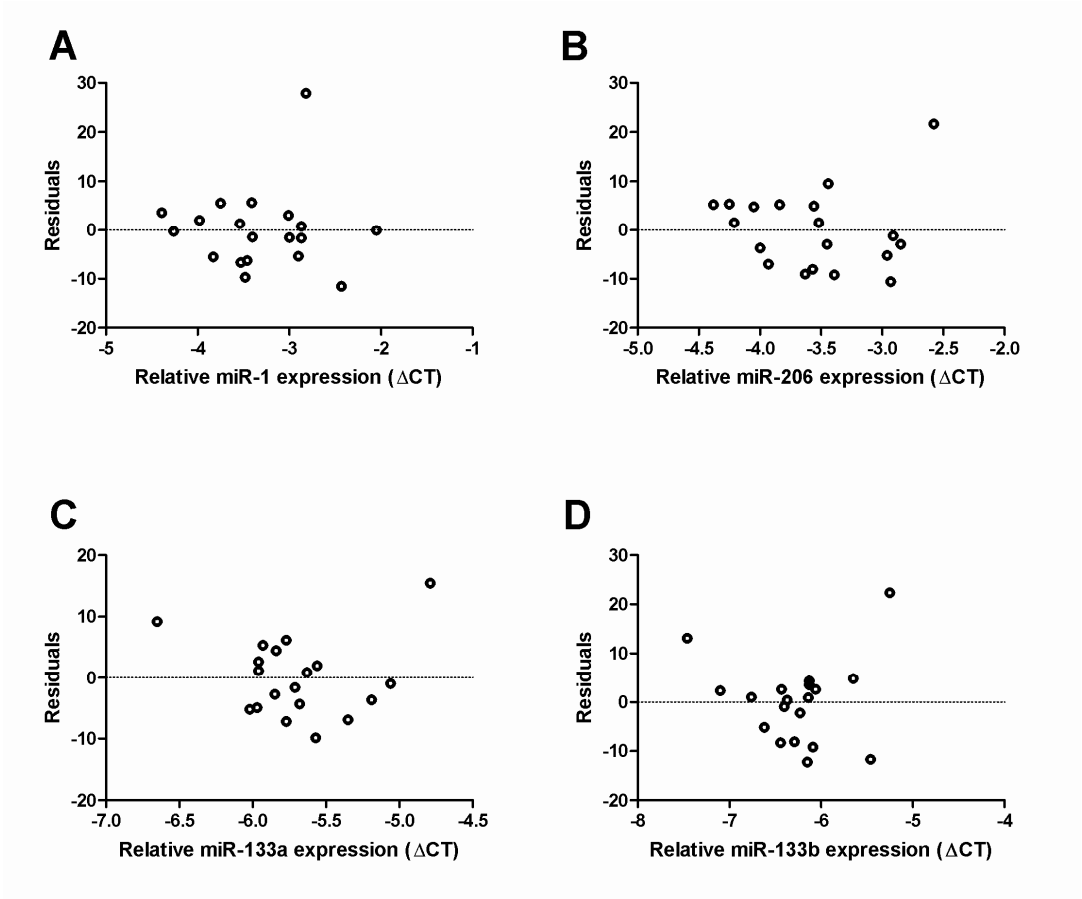
Supplementary Figure 3-3. RNU48 expression was not significantly different between control patients (0% weight loss), pre-cachexia patients (0-10% weight loss) and cachexia patients (>10% weight loss) (n=58). Data presented as CT ± SE.



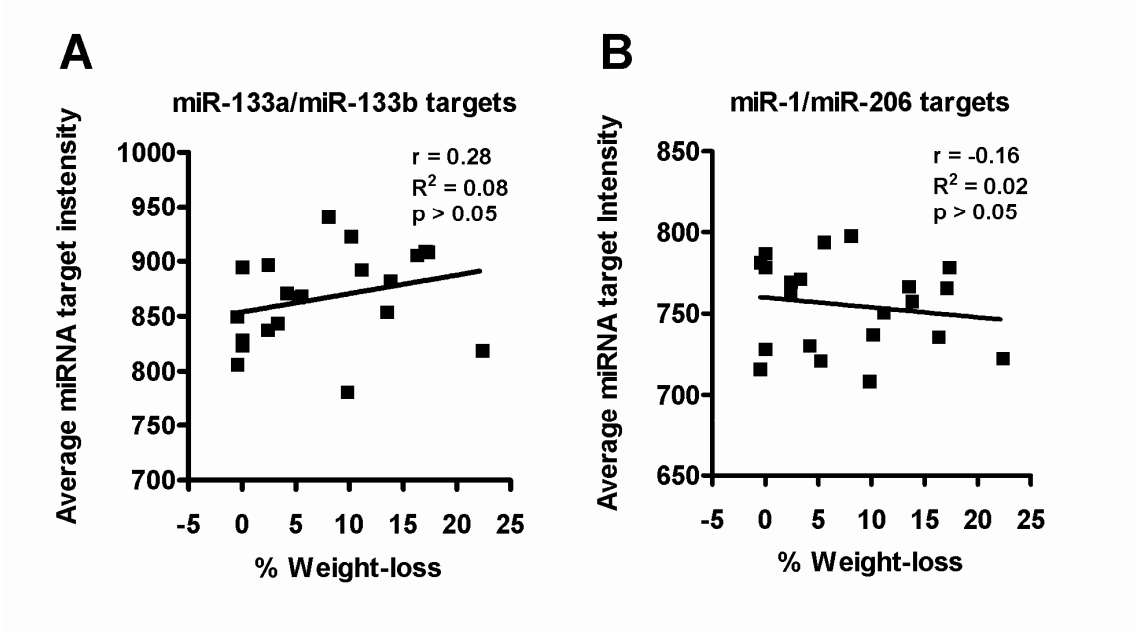
Supplementary Figure 3-4. Efficiency of pri-microRNA primers targeting (A) TUB1 to pri-miR-21, (B) TMEM49 to pri-miR-21 and (C)TMEM49 (host gene) across a range of cDNA dilutions.



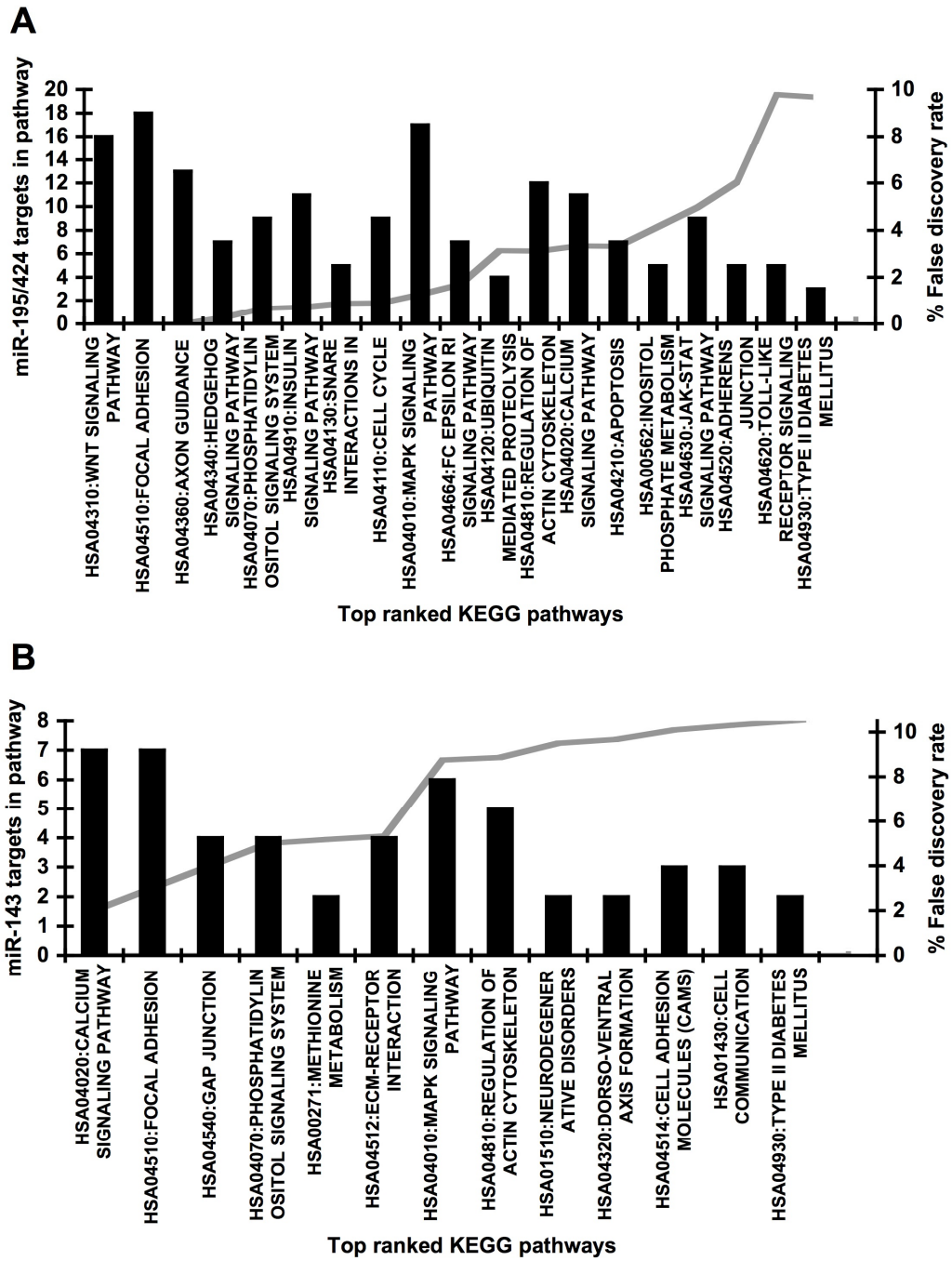
Supplementary Figure 3-5. (A) Correlation of miR-1, (B) miR-206, (C) miR-133a and (D) miR-133b expression with weight loss in cancer cachexia patients (n=58). Light-coloured markers represent healthy controls, dark-coloured markers represent cachexia patients. Regression lines were determined based on cachexia patients only, excluding controls.



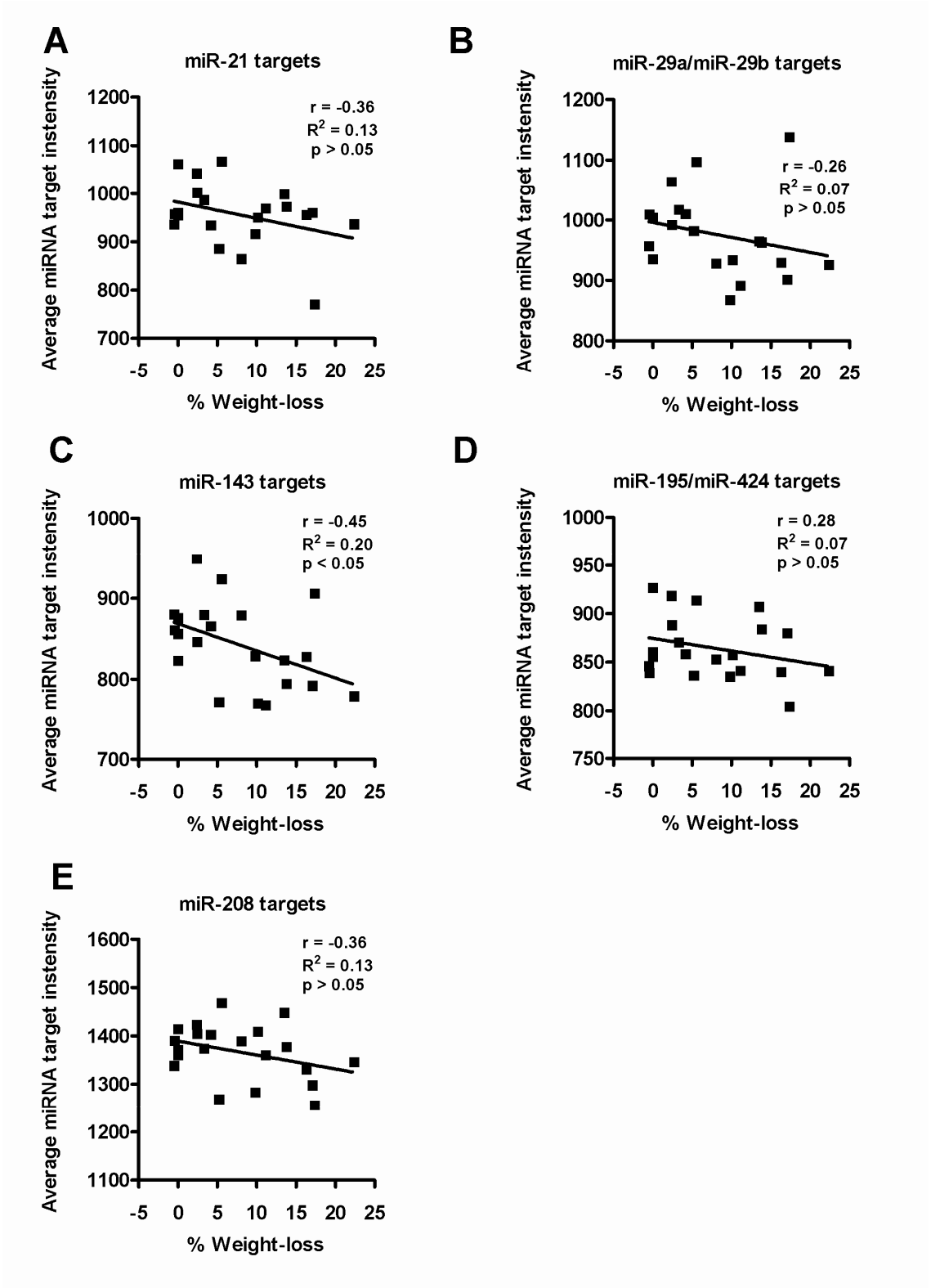
Supplementary Figure 3-6. Residual plots from multiple regression model predicting weight loss in pancreatic cancer patients based on (A) miR-1, (B) miR-206, (C) miR-133a and (D) miR-133b expression.



Supplementary Figure 3-7. Association of (A) miR-133a/miR-133b and (B) miR-1/miR-206 target expression with weight loss in patients (n = 20).



Supplementary Figure 3-8. Top ranked KEGG pathways enriched with (A) miR-195/424 targets and (B) miR-143 targets. Data shows number of microRNA targets (black-bars) and false discovery rate (grey-line) based on pathway enrichment analysis.



Supplementary Figure 3-9. Association of (A) miR-21, (B) miR-29a/miR-29b, (C) miR-143, (D) miR-195/miR-424 and (E) miR-208 target expression with weight loss in patients (n = 20).

Chapter 4 - Skeletal muscle microRNAs in Type 2 diabetes patients

4.1. Introduction

Type 2 diabetes is characterised by skeletal muscle insulin resistance and reduced glucose uptake in insulin sensitive tissues leading to hyperglycaemia (Muoio & Newgard, 2008). Type 2 diabetes is a major public health problem as it is estimated to affect over 170 million people worldwide (WHO, 2006) and by 2030 diabetes is projected to affect 4.4% of the world population (Wild et al. 2004). Type 2 diabetes is a leading cause of death, with over 8% of deaths in the US, Canada and the Middle East attributable to diabetes and worldwide it is the fifth leading cause of death (Roglic et al. 2005). Unfortunately, Type 2 diabetes can have mild symptoms, thus may only be detected several years after onset by which time further complications may have occurred (WHO, 2006). For example, Type 2 diabetes patients are at increased risk of microvascular and macrovascular complications including retinopathy, nephropathy, heart disease and stroke (WHO, 2006). Currently, impaired fasting glucose and impaired glucose tolerance are recognised as significant risk factors in Type 2 diabetes (Edelstein et al. 1997). However, the aetiology of Type 2 diabetes is still not fully understood, thus further research is needed to better understand the mechanism underlying the development of Type 2 diabetes and to identify early biomarkers of Type 2 diabetes.

Insulin resistance contributes to impaired glucose tolerance and individuals with impaired glucose tolerance (IGT) are at high risk of developing Type 2 diabetes but this is not inevitable (Edelstein et al. 1997; Söderberg et al. 2004). An eleven-year follow-up of individuals with IGT found only 30% developed Type 2 diabetes, while 30% reverted to normal glucose tolerance (NGT) (Söderberg et al. 2004). Decline in insulin secretion is also a well-recognised factor in the development of Type 2 diabetes. Pancreatic β-cell failure in Type 2 diabetes may be partly due to genetics, but may also precede the development of insulin resistance due to additional stress on the β-cells caused by decreased skeletal muscle insulin-stimulated uptake and hyperglycaemia that can be associated with insulin resistance (Muoio & Newgard, 2008). Therefore, focusing on understanding the underlying mechanisms of insulin resistance in skeletal muscle may help provide useful early biomarkers of T2D. Other clinical biomarkers associated with Type 2 diabetes include plasma TNFα, adiponectin, triacylglycerol and

HDL, which are reported to affect muscle insulin sensitivity and glucose uptake (Muoio & Newgard, 2008). However, none of these clinical biomarkers have been included in the WHO criteria for Type 2 diabetes diagnosis. Therefore more work to identify early biomarkers of Type 2 diabetes is required. Insulin signaling is an important modulater of muscle insulin sensitivity and defects in the insulin signaling cascade may contribute to insulin resistance and the development of Type 2 diabetes.

4.1.1 Insulin signaling and muscle glucose uptake

Insulin stimulates muscle glucose uptake by activating the insulin signaling cascade (Figure 2-2), which triggers translocation of the glucose transporter GLUT4 to the plasma membrane. Defects in the activation of the insulin signaling cascade may decrease insulin-stimulated glucose uptake, resulting in additional stress on the pancreatic β-cells to maintain normoglycaemia. Impairment of insulin signalling at multiple points has been reported, including impaired phosphorylation of IRS1, PI3K and AS160 (Kim et al. 1999; Kim et al. 2003; Karlsson et al. 2005; Cohen, 2006). Therefore, identifying regulators of insulin signaling which control skeletal muscle glucose uptake has generated much research interest (Cohen, 2006; Muoio & Newgard, 2008). MicroRNAs are post-transcriptional regulators of gene expression and are predicted to target genes coding for proteins important for insulin signaling and glucose uptake, but the role of microRNAs in Type 2 diabetes patients is largely unknown (Gauthier & Wollheim, 2006; Hennessy & O'Driscoll, 2008; Poy et al. 2007; Tang et al. 2008).

4.1.2 MicroRNAs target insulin signaling proteins

Several studies now suggest microRNAs may regulate components of the insulin signaling pathway (Figure 2-2) and thus may be important regulators of insulin resistance and Type 2 diabetes (Gauthier & Wollheim, 2006; Hennessy & O'Driscoll, 2008; Poy et al. 2007; Tang et al. 2008). For example, miR-29 has been shown to be elevated in adipose and muscle tissue of diabetic rats (He et al. 2007). Furthermore, overexpression of miR-29 was found to suppress insulin-stimulated glucose uptake in adipocytes (He et al. 2007). The insulin signaling proteins IRS1 and Akt were not directly repressed by miR-29, but IRS1 and Akt phosphorylation was reduced (He et al. 2007). Hyperglycaemia and hyperinsulinaemia were both reported to mimic miR-29 suppression of insulin-stimulated glucose uptake (He et al. 2007). However, it was not clear whether miR-29 was a cause or an effect of the hyperglycaemia and

hyperinsulinaemia observed in the diabetic mice (Hennessy & O'Driscoll, 2008; He et al. 2007).

Multiple microRNAs are predicted to target GLUT4 but few have been confirmed. Knockdown of miR-143 was found to inhibit expression of GLUT4 and PPAR-2 (Esau et al. 2004), yet miR-143 is not predicted to target GLUT4 or PPAR-2 (Lewis et al. 2003). Knockdown of miR-143 was associated with elevation in ERK5 protein and ERK is part of the MAPK signaling pathway, which is also activated by insulin via GRB2, SOS and RAS proteins. Post-exercise ERK activity is reported to be elevated (Yu et al. 2001) and activation of ERK appears to be correlated with *in-vitro* muscle glucose uptake (Babraj et al. 2009). However, inhibition of ERK was found to have no effect on insulin-stimulated glucose uptake (Cusi et al. 2000). It is not yet clear by which mechanism miR-143 affects GLUT4 or whether it is a regulator of muscle glucose uptake and involved in Type 2 diabetes.

Other potential microRNA biomarkers in Type 2 diabetes include miR-145. IRS1 is reportedly decreased in response to overexpression of miR-145 (Shi et al. 2007), which suggests that elevation of miR-145 could lead to impaired insulin signaling. However, miR-145 has not been confirmed to target IRS1 in skeletal muscle and miR-145 has not been previously associated with Type 2 diabetes (Hennessy & O'Driscoll, 2008).

4.1.3 MicroRNAs may target negative regulators of insulin signaling

MicroRNAs may also target negative regulators of the insulin signaling pathway. For example, the 3'UTR of protein tyrosine phosphatase PTP1B contains predicted binding sites for miR-1, miR-206 and miR-29 (Lewis et al. 2003). PTP1B is a negative regulator of insulin signaling and can reverse phosphorylation of the insulin receptor (Galic et al. 2005; Muoio & Newgard, 2008). In addition, inhibitors of PTP1B can increase insulin sensitivity in mice (Erbe et al. 2009). Furthermore, a report suggests Type 2 diabetes susceptibility may be increased by single nucleotide polymorphisms in the 3'UTR of PTP1B (Bento et al. 2004). Taken together these studies suggest microRNAs may also target negative regulators of insulin signalling. Therefore, loss of microRNA expression may lead to a decrease in translational repression and inhibition of insulin signaling. However, the predicted microRNA target sites of these negative insulin signaling regulators have yet to be confirmed in skeletal muscle.

In skeletal muscle, miR-1, miR-206 and miR-133 are highly expressed during development and are modulated in response to different cellular stresses (Chen et al. 2006; van Rooij et al. 2008; Chen et al. 2009). These microRNAs are modulated during hypertrophy, atrophy and during remodelling of skeletal muscle (McCarthy et al. 2007; Allen et al. 2009; McCarthy & Esser, 2007; Drummond et al. 2008). In Chapter 3, miR-1, miR-206 and miR-133 appeared to be down-regulated in skeletal muscle from pancreatic cancer patients in proportion to weight loss. Cancer patients are also reported to develop skeletal muscle insulin resistance and therefore are at increased risk of developing Type 2 diabetes (Rofe et al. 1994; Heber & Tchekmedyian, 1992). Unfortunately, it could not be established in Chapter 3 whether miR-1, miR-133 and miR-206 down-regulation was directly linked to insulin resistance. However, pathway enrichment analysis of miR-1, miR-133 and miR-206 targets suggested these microRNAs might target genes involved in insulin signaling and relevant to Type 2 diabetes.

4.1.4 Evidence of transcriptional changes in Type 2 diabetes skeletal muscle

The evidence suggests microRNAs could be important in the regulation of genes involved in insulin resistance and Type 2 diabetes (Hennessy & O'Driscoll, 2008; Gauthier & Wollheim, 2006; Poy et al. 2007; He et al. 2007; Tang et al. 2008). The established mechanism of microRNA action is via post-transcriptional mRNA cleavage or translational repression (Bartel, 2009). Previously genome-wide studies of transcriptional changes in insulin resistance and Type 2 diabetes suggested down-regulation of PGC1-α and reduced expression of oxidative phosphorylation may be responsible for the insulin resistance in Type 2 diabetes (Mootha et al. 2003; Patti et al. 2003). However these studies appear to be confounded by the effect of BMI and VO_2max on expression of oxidative phosphorylation associated genes. Both BMI and VO_2max have been shown to influence PGC-1α and oxidative phosphorylation genes (Krämer et al. 2006; Ling et al. 2004; Mathai et al. 2008; Sriwijitkamol et al. 2007; Timmons et al. 2006). A more recent genome-wide transcriptome study found no evidence of transcriptional changes in myotubes from Type 2 diabetes patients when compared to BMI and physical activity matched controls (Frederiksen et al. 2008). In addition, a study from our laboratory (J. Timmons, personal communication) compared genome-wide transcript profiles (n = 118) from skeletal muscle of Type 2 diabetes patients, IGT patients and healthy controls matched by age, BMI and VO_2max. There was no evidence of any global transcriptional changes between Type 2 diabetes, IGT or

healthy skeletal muscle (J. Timmons, personal communication), supporting the view that a post-transcriptional mechanism may be involved in the development of insulin resistance and the pathogenesis of Type 2 diabetes (Hennessy & O'Driscoll, 2008; Poy et al. 2007; Tang et al. 2008). Therefore, microRNAs are possible post-transcriptional regulators of insulin resistance and Type 2 diabetes or alternatively microRNAs may be useful early biomarkers of Type 2 diabetes.

4.1.5 Aims

- Determine whether skeletal muscle expression of miR-1, miR-133a, miR-133b and miR-206 is changed in Type 2 diabetes patients.
- Determine whether skeletal muscle microRNA transcription or processing is altered in Type 2 diabetes patients.
- Examine whether skeletal muscle expression of predicted microRNA targets changes in Type 2 diabetes patients.
- Identify further microRNAs based on experimental validation of microRNA array data, which may be early biomarkers of Type 2 diabetes.

4.2. Methods

4.2.1 Subjects

Thirty non-obese individuals were selected from a larger Scandinavian Type 2 diabetes cohort (n = 215) and assigned into three groups based on glucose tolerance: Type 2 diabetes (n = 10), impaired glucose tolerance (IGT; n = 10) and controls with normal glucose tolerance (NGT, n = 10). These groups were matched on age, BMI and maximal oxygen uptake (VO_2max). Exclusion criteria were as follows: cardiovascular disease associated with claudication, cerebrovascular accidence, angina pectoris, prior coronary artery bypass graft, prior percutaneous transluminal coronary angioplasty, insulin treatment, recent infection and/or history of malignant disease. Patients received information about the experimental procedures before giving their written informed consent. The Type 2 diabetes patients were instructed to stop hypoglycaemic medication one week prior to the study. The study was approved by the Ethics Committee of Copenhagen and Frederiksberg Council (01-141/04) and the analysis was approved by the Ethics committee of the School of Life Sciences, Heriot-Watt University, Edinburgh.

Physical characteristics of the patients are summarised in Table 4-1. These patients have been included in a previous larger case-controlled study (Krabbe et al. 2007; Plomgaard et al. 2007; Nielsen et al. 2008). There were no significant differences between patients for age, BMI or VO_2max ($P > 0.05$).

Table 4-1. Physical characteristics of patients

Patient characteristics	Type 2 diabetes (n = 10)	IGT (n = 10)	Controls (n = 10)
Age (y)	60.4 ± 14	60.2 ± 7.1	60.3 ± 7.5
Weight (kg)	85.6 ± 12.2	81.1 ± 8.2	78.4 ± 8.9
Height (cm)	179 ± 10	176 ± 6	174 ± 9
BMI	26.6 ± 1.9	26.3 ± 1.7	25.9 ± 1.9
VO_2max (ml/kg/min)	28.8 ± 8.6	29.4 ± 6.9	28.4 ± 6.0

Data are presented as mean ± SD.

4.2.2 Approach

Muscle specific mature microRNAs, miR-1, miR-133a, miR-133b and miR-206 were profiled in skeletal muscle biopsies from Type 2 diabetes, IGT and control patients using RT-qPCR (see section 4.2.6), followed by examination of primary microRNAs (see section 4.2.8), precursor microRNAs (see section 4.2.10) and expression of microRNA processing genes to identify if there were any alterations in muscle-specific microRNA transcription or processing in Type 2 diabetes. The predictive power of patient microRNA expression was tested using a multiple regression approach. The predicted targets of differentially expressed microRNAs were analysed further as a group to determine their possible biological, cellular and molecular role in Type 2 diabetes pathogenesis (see section 4.2.13). Existing microarray data on mRNA changes in response to microRNA overexpression were mined for links to the insulin resistance and Type 2 diabetes literature (see section 4.2.11). To identify further new potential Type 2 diabetes candidate microRNAs, microRNA target expression signatures were calculated for each patient based on Type 2 diabetes microarray data from our laboratory (see section 4.2.12). These microRNA target expression signatures were analysed by SAM to identify microRNA target signatures differentially expressed in Type 2 diabetes patients. Finally, experimental validation of microRNA array data was conducted to confirm differential expression of microRNAs using RT-qPCR (see section 0).

4.2.3 Muscle biopsy and clinical examination

The patients reported to the hospital for a clinical health examination and an oral glucose tolerance test between 0800h and 1000h after an overnight fast. Muscle biopsies were obtained from the Vastus lateralis using the Bergstrom needle technique with suction under basal condition. Biopsies were quickly dissected free from visible blood and connective tissue, frozen in liquid nitrogen and stored at -80°C until RNA extraction. An oral glucose tolerance test was performed according to World Health Organization guidelines (WHO, 2006). Patients consumed 75 g glucose in 500 ml water, blood samples were taken at baseline, 1 and 2 h. Patients were classified as NGT, IGT or Type 2 diabetes based on the WHO criteria (WHO, 2006). NGT was defined as fasting plasma glucose <7.0 and plasma glucose <7.8 mmol/l after OGTT; IGT was defined as fasting plasma glucose <7.0 mmol/l and plasma glucose between 7.8-11.0 mmol/l OGTT; Type 2 diabetes was defined as fasting plasma glucose >6.9 mmol/l or plasma glucose >11.0 mmol/l after OGTT. BMI was calculated as body mass

(kg) / body height $(m)^2$. Cardiorespiratory fitness was measured using the Åstrand-Rhyming indirect test of maximal oxygen uptake (VO_2max) (Astrand & Ryhming, 1954).

4.2.4 Blood analysis

Blood samples collected into glass tubes with EDTA were immediately spun at 3500g for 15 min at 4°C and plasma was stored at −20°C until further analysis. Plasma TNF-α, sTNFR2 and IL-6 were measured by colleagues in duplicate using ELISA (R&D Systems, USA) (Plomgaard et al. 2007). Plasma cholesterol (HDL and LDL), triacylglycerol, C-reactive protein (CRP), glucose and insulin were measured using routine laboratory methods as previously described (Plomgaard et al. 2007). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA[IR]; software available at http://www.dtu.ox.ac.uk).

4.2.5 RNA isolation, quantification and screening for RNA purity

Muscle biopsies were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) using a motor-driven homogenizer (Polytron, Kinematica, Newark, NY, USA) and total RNA was isolated according to the manufacturer's protocol. The size of the tissue samples ranged between 10 and 40 mg. Total RNA was dissolved in 30 µl RNase-free water, quantified using a Spectrophotometer (Pharmacia Biotech, NJ, USA) as previously described (Plomgaard et al. 2007), then stored at -80°C prior to analysis.

Later all RNA from the whole diabetes cohort (n = 215) was re-quantified using a Nanodrop spectrophotometer (Thermoscientific, UK). The Nanodrop measures absorbance in RNA samples (range 200 to 350 nm) and requires <2 µl solution. RNA quantification is based on absorbance at 260 nm. Additional absorbance around 220-230 nm indicates presence of contaminants in the samples, including salts such as guanidinium isothiocyanate and phenol or proteins. Phenol contamination can affect absorption at 270 nm and therefore can cause spurious RNA quantification. The 260/280 ratio should ideally be >2.0 indicating RNA free of contaminants. The 260/230 ratio was <2 indicating possible phenol or guanidinium isothiocyanate contamination from the RNA isolation procedure. Phenol contamination can lead to underestimation of RNA quantity and affect downstream measurements including RT-qPCR via inhibition of Taq polymerase and reduced primer efficiency (Fleige & Pfaffl, 2006). Unfortunately, although the Nanodrop is useful for RNA quantification and

screening for contaminants, it provides no indication of RNA integrity. To limit possible effects a subset of patient RNA samples were re-precipitated at -20°C overnight in 2.5 x RNA volume 100% ethanol and 0.5 x RNA volume 7.5 M sodium acetate. Following re-precipitation of a subset of RNA samples the 260/230 ratio improved to 1.5-2.0 and increased RNA yield (Supplementary Table 4-3). Therefore, the decision was made to reprecipitate and re-quantify all RNA samples from the Type 2 diabetes cohort (n = 215) using the Nanodrop spectrophotometer (data not shown). Comparison of the inter-laboratory determined RNA concentrations revealed good concordance between laboratories despite the use of different spectrophotometers, although there was one outlier (Supplementary Figure 4-9). RNA quality was assessed prior to microRNA expression analysis (n = 30) with the Agilent Bioanalyser using the procedure described in 3.2.4. RIN scores >8 indicated RNA quality was high with no sign of RNA degradation on the micro-electrophoresis gel (Supplementary Figure 4-8).

4.2.6 Mature-microRNA reverse transcription and real-time quantitative PCR

Reverse transcription (RT) of RNA to cDNA was conducted using the TaqMan® MicroRNA Reverse Transcription Kit according to the manufacturer's instructions. Each RT reaction was set up to contain between 2-10 ng of total RNA. The RT reaction was prepared as described in section 3.2.5. TaqMan® MicroRNA Assays were used to measure miR-1, miR-133a, miR-133b, miR-206 and RNU48 listed previously in Chapter 3, Table 3-2. Each PCR reaction was prepared as previously described in section 3.2.6. The PCR reaction was run on an Applied Biosystems 7300/7500 Fast Real-Time PCR system in 9600 emulation mode. Ct values for triplicates were averaged, and ΔCt values computed using RNU48 as the endogenous control. Comparison of average RNU48 Ct between Type 2 diabetes, IGT and control groups indicated RNU48 expression was not significantly difference between groups (Supplementary Figure 4-1). Fold change was calculated using the -2ΔΔCT method (Schmittgen & Livak, 2008).

4.2.7 Design and validation of pri-microRNA primers

To determine whether pri-microRNA transcription was regulated in Type 2 diabetes, primers were designed to amplify the intronic region between the pre-microRNA hairpin and the predicted host gene (Figure 4-1). C20orf166 is the predicted host gene of pri-miR-1-1 and pri-miR-133a-2, primers were designed to span exon 2-3 of C20orf166. ENSESTG00000007014 is located adjacent to pri-miR-133a-1 and pri-miR-

1-2, primers were designed to amplify exon 2 of ENSESTG00000007014. All primers for pri-microRNA transcripts are listed in Table 4-2. Primers were obtained from Invitrogen UK.

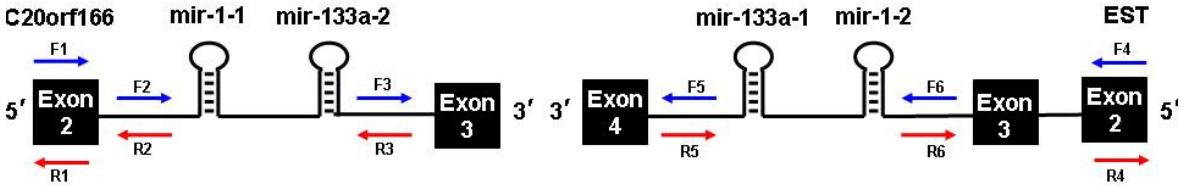


Figure 4-1. Location of primers designed to amplify pri-miR-1 and pri-miR-133a transcripts. Primers were also designed to amplify the predicted host genes. Arrows indicate location of forward and reverse primers.

Primer efficiency was tested on five serial dilutions of cDNA using the RT-qPCR protocol described in section 4.2.9. Ct values were found to be linear for pri-miR-1-1, pri-miR-1-2, pri-miR-133a-1 and pri-miR-133a-2, with the amplification slope between 3 and 3.2 indicating similar amplification efficiency for these primers (Supplementary Figure 4-11). However, standard curves for the predicted host genes C20orf166 and ENSESTG00000007014 values were not linear, so these primers were not used (Supplementary Figure 4-11). In addition, melting curves were examined to determine whether a single PCR product was formed (not presented) and PCR products were analysed on a 2% agarose gel which was run as follows: 2% agarose (0.8g) was dissolved in 0.5% TBE by heating for 2 min and 0.5 µl DNA bromide red dye was added. The gel was poured and left to polymerise for 10 min. In the 1st well, low weight DNA ladder was loaded, 2 µl of PCR product were mixed with 1 µl blue dye and loaded on gel. The gel was run at 100V for ~1 h in a running buffer of 0.5% TBE. Afterwards, DNA bands were visualised under UV light to confirm a single PCR product.

Table 4-2. Primer sequences to amplify pri-miR-1 and pri-miR-133a transcripts.

Target	Identifier	Primers, sequences or probes
Pri-hsa-miR-1-1	ENSG00000199017	5'-caggcgctcgagactttct-3' (forward)
		5'-tcacacactcacacgatcca-3' (reverse)
Pri-hsa-miR-133a-2	ENSG00000207764	5'-tctatcctatggctcacaaaagc-3' (forward)
		5'-ctcactcacgggtggaaac-3' (reverse)
Pri-hsa-miR-133a-1	ENSG00000207786	5'-aaatgtactttctgtgactgaggtgt-3' (forward)
		5'-ctgtgggcaaaaggagacat-3' (reverse)
Pri-hsa-miR-1-2	ENSG00000207694	5'-aagttgttagctgtaaaaacatgaaa-3' (forward)
		5'-ttcgataaattagtctctgcaaatg-3' (reverse)

Primers were obtained from Invitrogen, UK.

4.2.8 Pri-microRNA reverse transcription

RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, UK). The RT master mix was prepared with 4 µl 10 x RT buffer, 1.6 µl 25 x dNTP mix (100 mM), 4 µl 10 x RT random primers, 2 µl MultiScribe reverse transcriptase, 2 µl RNase inhibitor and 6.4 µl Nuclease-free water. RNA was diluted to 2 µg/µl in 20 µl of Nuclease-free water. The RT reaction was prepared by mixing 20 µl RT mastermix, 20 µl RNA sample and spinning down. The thermal cycler was programmed to run at 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec and then held at 4°C. cDNA samples were stored at -20°C until further analysis.

4.2.9 Pri-microRNA real-time quantitative PCR

SYBR green reagents (Applied Biosystems, UK) were used for RT-qPCR reaction to amplify regions spanning pri-microRNA transcripts; the primers used are listed in Table 4-2. Each RT-qPCR reaction was prepared using 6 µl SYBR green mastermix, 4.6 µl Nuclease-free water, 0.10 µl forward primer, 0.10 µl reverse primer and 1.2 µl cDNA (1:10 dilution). For each primary microRNA transcript all patient PCR reactions were prepared in triplicate on the same 384 well plate. Triplicate wells containing no template were also prepared as an additional control to indicate if PCR contaminants were present (Bustin & Nolan, 2004). The plate was sealed and spun down at 3000 rcf in a centrifuge at 4°C. The PCR reaction was run on an Applied Biosystems 7300/7500 Fast Real-Time PCR system in standard mode. The following thermal cycling parameters were applied, 2 min at 50°C, 10 min at 95°C, then 45 cycles consisting of 15

s at 95°C and 60 s at 60°C. Ct values for triplicates were averaged, and ΔCt values computed using 18S as the endogenous control. Comparison of the average Ct of 18S between Type 2 diabetes, IGT and control groups indicated 18S expression was not significantly difference between groups (Supplementary Figure 4-3). Fold change was calculated using the -2ΔΔCT method (Schmittgen & Livak, 2008).

4.2.10 Pre-microRNA detection by Northern blot

Northern blot was performed to determine whether pre-miR-133a was altered in Type 2 diabetes, IGT and control groups. RNA (10 ng) was pooled from patients with diabetes, impaired glucose tolerance or normal glucose tolerance respectively. In addition, four individual patient samples were used, including two Type 2 diabetes patients and two controls. RNA samples were precipitated overnight in 100% ethanol and then centrifuged at 14000rpm for 30 min at 4ºC. The RNA pellet was washed in 75% ethanol and redissolved in 6 µl of 2 x formide loading dye. A small RNA blot was prepared using 15% denaturing gel, consisting of 15 ml SequaFlowGel sequencing system concentrate, 7.5 ml SequaFlowGel diluent, 2.5 ml 10 x MOPS buffer, 250 µl 10% Ammonium persulfate (APS, Sigma) and 25 µl TEMED. Polymerization was completed in 10 min, wells were washed in 1 x MOPS running buffer. The gel was preheated by running at 100V for 30 min in the WB system (Invitrogen), 1 x MOPS/NaOH (20mM, pH 7.0) was used as the running buffer. In the meantime, RNA samples were incubated at 95ºC for 2 min and then loaded on to the gel with a Decade Marker (AM7778, Applied Biosystems). The gel was run at 100 V for 3 h and the running buffer was changed after 1.5 h.

The RNA was transferred to blot at 400 mA for 1-1.5 h, using a neutral membrane (HybondN, Amersham Biosciences) and gel blotting paper. For chemical cross-linking, the blot was incubated at 55ºC for 2 h, the blot was submerged in a cross linking reagent consisting of 9 ml RNase free water, 245 µl 1-methylimidazole, 300 µl 1M HCl, 0.753 g EDC and Milipore water upto 24 ml. The blot/membrane was incubated at 37ºC for 1 h in a prehydridisation mix. This consisted of 12.5 ml formamide, 6.25 ml SSPE (20 x), 1.25 ml denhardt (100 x), 1.25 ml 10% SDS and 500 µl hsDNA (2 mg/ml) which was denatured at 95ºC for 2 min before adding to the blot/membrane.

A hydridisation mix was prepared separately consisting of 1 µl 50 uM oligonucleotide, 11 µl nuclease-free water, 2 µl, 10x buffer, 2 µl RNase inhibitor, 2 µl T4 PNK and 2 µl

32P-j-ATP. The hydridisation mix was spun down and then incubated at 37ºC for 1 h. After this 20 µl nuclease-free water was added and the hybridisation mix was filtered with a G-25 column, which was then transferred to the pre-hybridisation mix. The blot was hybridised over night at 37ºC, washed twice with 2 x SSC and 0.1% SDS for 1.5 h at 65ºC. Exposition was completed overnight. The membranes were stripped with 1 L stripping solution for 10-15 min at 100ºC. The stripping solution consisted of 10 ml 1M Tris (pH 8.5), 10 ml 0.5M EDTA and 10 ml 10% SDS, made up to 1L with Millipore water. Blots were reprobed for t-RNA, which was used as a control. Image J was used to analyse blots, signals were background corrected then normalised to t-RNA.

4.2.11 Analysis of microRNA targets associated with Type 2 diabetes

Existing microarray data (GSE8501) from HeLa cells 24 h following transfection of miR-133a allowed examination of potential miR-133a regulation of diabetes-associated targets. This approach can provide indirect validation of microRNA targets. In the present study miR-133a targets with previous links to the diabetes literature were manually curated based on citations in the NCBI database. Microarray data (GSE8501) was retrieved from GEO (NCBI). The platform used to generate the microarray data was a non-commercial glass oligonucleotide array containing probes designed against >23000 human genes. Pre-processed log2 intensity data was available, so the difference in log2 intensity between transfected and control cells was calculated. Analysis of miR-133a target expression in response to miR-133a transfections was restricted to targets with >1 associated diabetes citations in the NCBI database.

4.2.12 Analysis of microRNA target signatures

Evidence of microRNA action on target mRNA cleavage was examined based on mean absolute microRNA target expression (Arora & Simpson, 2008). Unpublished microarray data was available from our laboratory from a larger Type 2 diabetes cohort (n=115), which was MAS 5.0 normalised and absent filtered data (I. Gallagher, personal communication). For each patient, average expression of targets for each of the human microRNAs was computed as described in section 3.2.10. Thus generating a microRNA target signature that could help reveal candidate microRNAs involved in Type 2 diabetes. Firstly, the microRNA target signatures for the muscle-specific microRNAs were calculated and differences between Type 2 diabetes, IGT and control groups were determined using one-way ANOVA.

Secondly, a more global approach was taken to find other potential candidate microRNAs involved in Type 2 diabetes. Significance Analysis of Microarrays (SAM) was used to identify differences in individual microRNA target signatures between groups. SAM was run using the siggenes package in Biconducter. SAM computes a moderated t-statistic to adjust for multiple testing and calculates a false discovery rate.

4.2.13 Gene ontology and pathway enrichment analysis

Gene ontologies (GO) were used to gain an overview of the possible biological, cellular and molecular functions of the differentially expressed microRNAs in Type 2 diabetes patients. The Expression Analysis Systematic Explorer (EASE) (Sherman et al. 2007) was used to determine which gene ontologies may be regulated by differentially expressed Type 2 diabetes microRNAs as described in section 3.2.11.

4.2.14 MicroRNA array experimental validation

MicroRNA array data from pooled patient RNA was analysed using Significance of Analysis of Microarray (SAM; http://www.stat.standford.edu/~tibs/SAM) to determine differentially expressed microRNAs (I. Gallagher, personal communication). RT-qPCR was used to measure the differentially expressed microRNAs including miR-27b, miR-29a, miR-29b, miR-143, miR-208 and miR-424 as described in section 3.2.6. The mature microRNA sequences and catalogue references are listed in Table 3-4.

4.2.15 Statistical analysis

Expression of mature microRNAs, primary microRNAs and microRNA processing genes was compared between Type 2 diabetes, IGT and control groups using one-way ANOVA assuming equal variances between groups based on Barletts test. Post-hoc Bonferroni tests were performed where necessary to identify inter-group differences. When the assumption of equal variances was not met the non-parametric Kruskal Wallis test was run. Linear regression was used to test whether individual patient microRNA expression could explain a significant proportion of the variance in clinical measures of insulin resistance and glucose homeostasis including, fasting glucose, fasting insulin, 2h glucose tolerance, HbA1c and HOMA[IR]. SAM was used to identify differentially expressed microRNA target signatures as described in 4.2.12. The Fishers Exact test was the basis for the gene ontology and pathway enrichment analysis as described in 4.2.13.

4.3. Results

Patients' glucose tolerance and insulin sensitivity are shown in Table 4-3. Fasting glucose, 2 h glucose tolerance and HbA1c were all significantly higher in the Type 2 diabetes compared to IGT and control group (P <0.01). HOMA[IR] was significantly higher in Type 2 diabetes compared to controls (P <0.01). Basal insulin was significantly elevated in the Type 2 diabetes patients compared to the healthy controls. Patient inflammatory markers are summarised in Supplementary Table 4-1, there were no significant differences between groups for CRP, IL-6, IL-15, IL-18, TNFα, and TNFαR2.

Table 4-3. Glucose homeostasis and insulin sensitivity markers.

Patient characteristics	Type 2 diabetes (n=10)	IGT (n=10)	Control (n=10)
Fasting glucose (mmol/L)	11.3 ± 2.9***	5.9 ± 0.5†††	5.0 ± 0.4
2-h glucose (mmol/L)	21.1 ± 5.1***	7.5 ± 1.8†††	5.1 ± 1.6
HbA1c (%)	8.3 ± 1.3***	5.8 ± 0.2	5.6 ± 0.3
Basal insulin (μU/ml)	76 ± 60*	56 ± 22†††	28 ± 12
HOMA[IR]	5.8 ± 3.9***	2.5 ± 0.9†	1.0 ± 0.5

Data are presented as mean ± SD.

* $P < 0.05$ when compared with the control group

** $P < 0.01$ when compared with the control group

*** $P < 0.0001$ when compared with the control group

† $P < 0.05$ when compared with the control group

†† $P < 0.01$ when compared with the control group

††† $P < 0.0001$ when compared with the control group

4.3.1 Down-regulated miR-133a and miR-206 expression in Type 2 diabetes

To determine whether muscle-specific miR-1, miR-133a, miR-133b or miR-206 was significantly altered in Type 2 diabetes, the mean fold change of these microRNAs was compared between Type 2 diabetes, IGT and control groups. There was no significant

difference in miR-1 expression between the Type 2 diabetes or the IGT group compared to the controls (Figure 4-2). In the Type 2 diabetes group miR-206 was significantly lower compared to the IGT ($P < 0.01$) and healthy control group ($P < 0.05$; Figure 4-2).

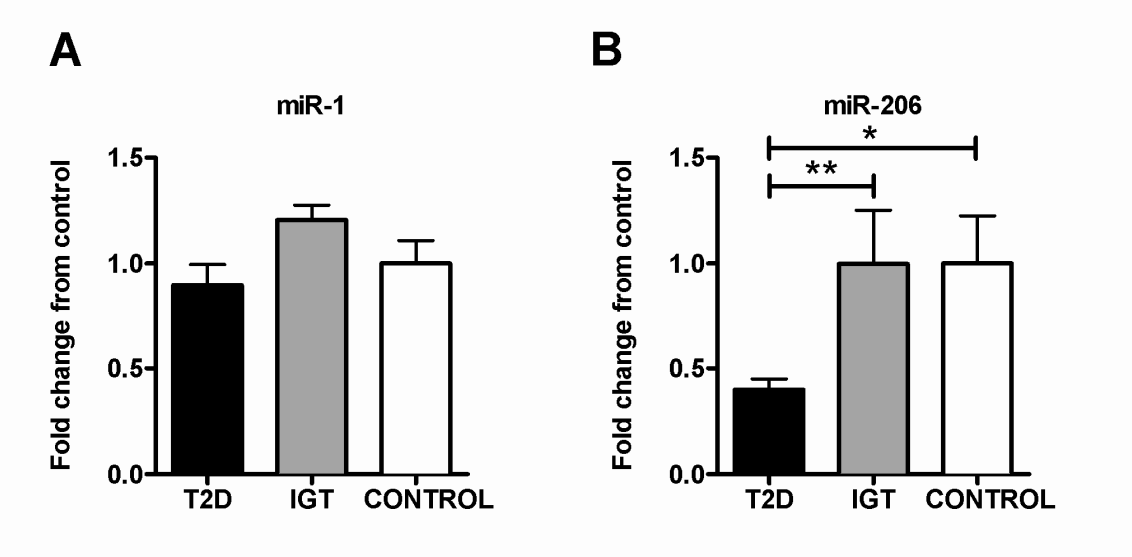


Figure 4-2. Fold change in (A) miR-1 and (B) miR-206 expression in skeletal muscle from patients with Type 2 diabetes, IGT compared to controls. Data shown as mean ± SE. * $P < 0.05$ ** $P < 0.01$

In the Type 2 diabetes patient group there was a significant down-regulation of miR-133a compared the IGT group ($P < 0.05$) and the control group ($P < 0.001$; Figure 4-3), but miR-133b remained relatively stable, with no significant differences in miR-133b detected between groups (Figure 4-3). The discordance in miR-133a and miR-133b expression raises the question whether there are significant differences in copy number of individual microRNA family members in skeletal muscle. Potentially certain microRNA family members could be more important in target repression. The relative abundance of a gene can be compared based on the cycle threshold, providing amplification efficiency and PCR reaction conditions are identical. Comparison of the cycle threshold of miR-133b and miR-133a suggested similar abundance in skeletal muscle (Supplementary Figure 4-2).

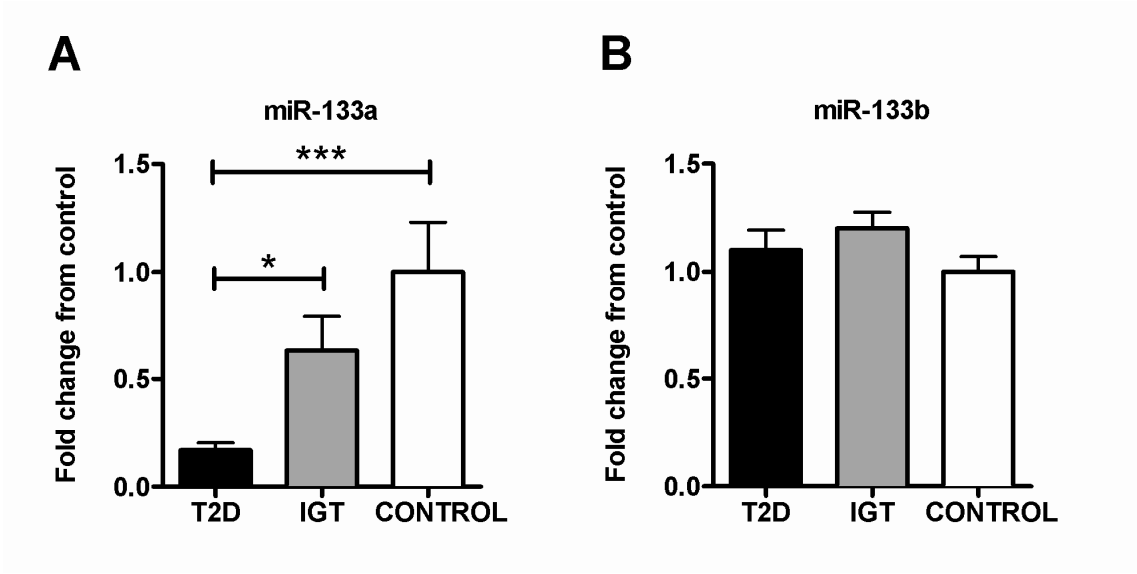


Figure 4-3. Fold change in (A) miR-133a and (B) miR-133b in skeletal muscle from patients with Type 2 diabetes, IGT compared to controls. Data shown as mean ± SE. * *P* < 0.001 * *P* < 0.05**

4.3.2 Transcription of miR-133a is unchanged in Type 2 diabetes

The significant down-regulation of mature miR-133a in Type 2 diabetes patients raises the question whether this is caused by decreased microRNA transcription or processing (see Chapter 2, section 2.2). To determine whether the mature miR-133a expression observed in Type 2 diabetes could be due to changes in microRNA transcription, primers were designed against the two known miR-133a transcripts, miR-133a-1 on chromosome 18 and miR-133a-2 on chromosome 20 (Figure 4-1). Both transcripts can be processed into identical mature miR-133a sequences, but there were no significant differences in pri-miR-133a-1 or pri-miR-133a-2 between the Type 2 diabetes, IGT and control group (Figure 4-4). In addition, there were no significant differences in pri-miR-1-1 or pri-miR-1-2 between the Type 2 diabetes, IGT and control group (Figure 4-4), which indicates a microRNA processing step downstream of primary transcription must be responsible for the down-regulation of miR-133a in Type 2 diabetes.

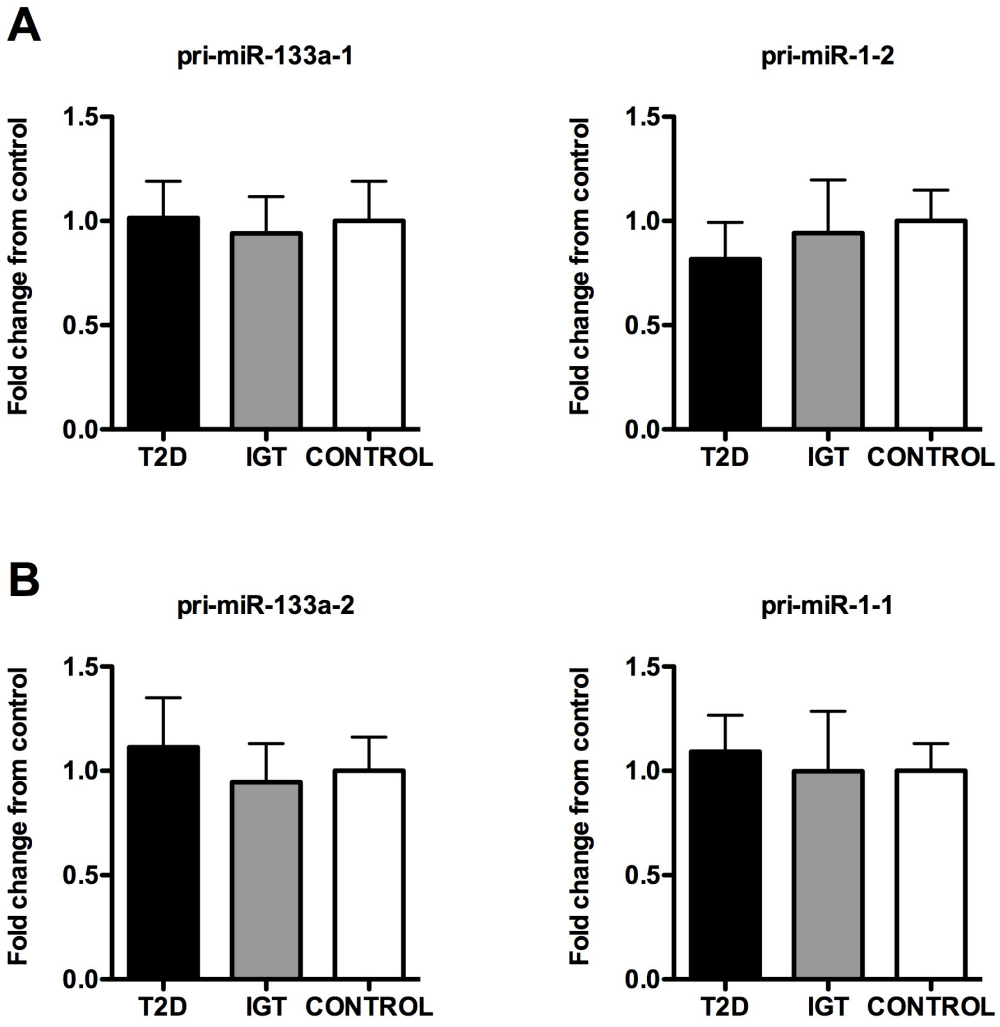


Figure 4-4. Expression of pri-microRNA transcripts encoding miR-1 and miR-133a. (A) Fold change in pri-miR-133a-1 and pri-miR-1-2 encoded together on chromosome 18. (B) Fold change in pri-miR-133a-2 and pri-miR-1-1 encoded together on chromosome 20 in skeletal muscle from patients with Type 2 diabetes, IGT or controls. Data shown as mean ± SE.

The current literature assumes miR-1 and miR-133a are transcribed together as two bicistronic transcripts (Chen et al. 2006). However, pri-miR-133a-2 had a significantly lower Ct compared to pri-miR-1-1 ($P < 0.001$; Figure 4-5) suggesting they may not be transcribed as a bicistronic transcript, or alternatively are processed at different rates. The ribosomal 18S gene was run as an endogenous control across Type 2 diabetes, IGT and control samples and was stably expressed across the groups (Supplementary Figure 4-3). Therefore the cycle thresholds presented in Figure 4-5 enable comparison of the absolute abundance of each of the primary microRNA transcripts. Pri-miR-133a-2 had

a significantly lower Ct than pri-miR-133a-1 ($P < 0.001$; Figure 4-5) suggesting it was more abundant, but this does not indicate whether pri-miR-133a-2 is primarily responsible for determining mature miR-133a levels. In contrast, pri-miR-1-1 and pri-miR-1-2 had similar cycle thresholds suggesting similar absolute abundance (Figure 4-5).

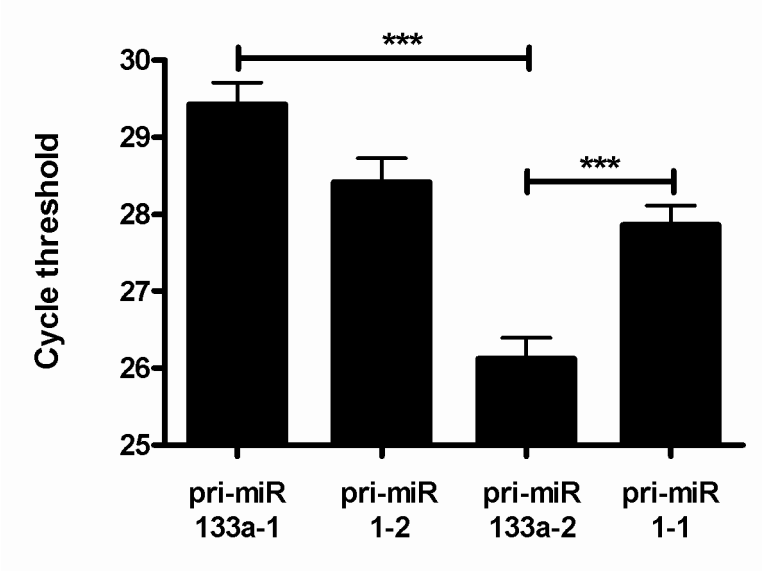


Figure 4-5. Absolute cycle threshold of pri-miR-133a-1, pri-miR-1-2, pri-miR-133a-2 and pri-miR-1-1 transcripts in skeletal muscle. Lower cycle threshold values indicate high transcript abundance. Data shown as mean ± SE. *** $P <$ 0.001

4.3.3 Pre-miR-133a expression in Type 2 diabetes

The lack of any difference in pri-miR-133a expression between Type 2 diabetes patients and controls, despite significant differences in mature miR-133a expression indicates a factor downstream of primary miR-133a transcription may be responsible for the observed down-regulation of mature miR-133a in Type 2 diabetes patients. Northern blot allows detection of both mature and pre-microRNA. The Northern showed that pre-miR-133a was substantially less abundant than mature miR-133a (Figure 4-6), most likely due to rapid processing in the cytoplasm by DICER, which is in accordance with previous pre-miR-133 expression data showing pre-miR-133a-1 and pre-miR-133a-2 were undetectable in skeletal muscle (Lee et al. 2008). Quantification of mature miR-133a expression levels from the Northern confirmed miR-133a was significantly down-regulated in Type 2 diabetes compared to controls (Supplementary Figure 4-4). The Northern appeared not to be as sensitive as RT-qPCR (see section 4.3.1), possibly because the probe against miR-133a may not have been able to differentiate between

miR-133 family members due to close sequence homology (Supplementary Table 4-2), so represents the average expression of miR-133a and miR-133b. In contrast, RT-qPCR appeared to be able to differentiate between miR-133a and miR-133b in the present study (Supplementary Figure 4-2).

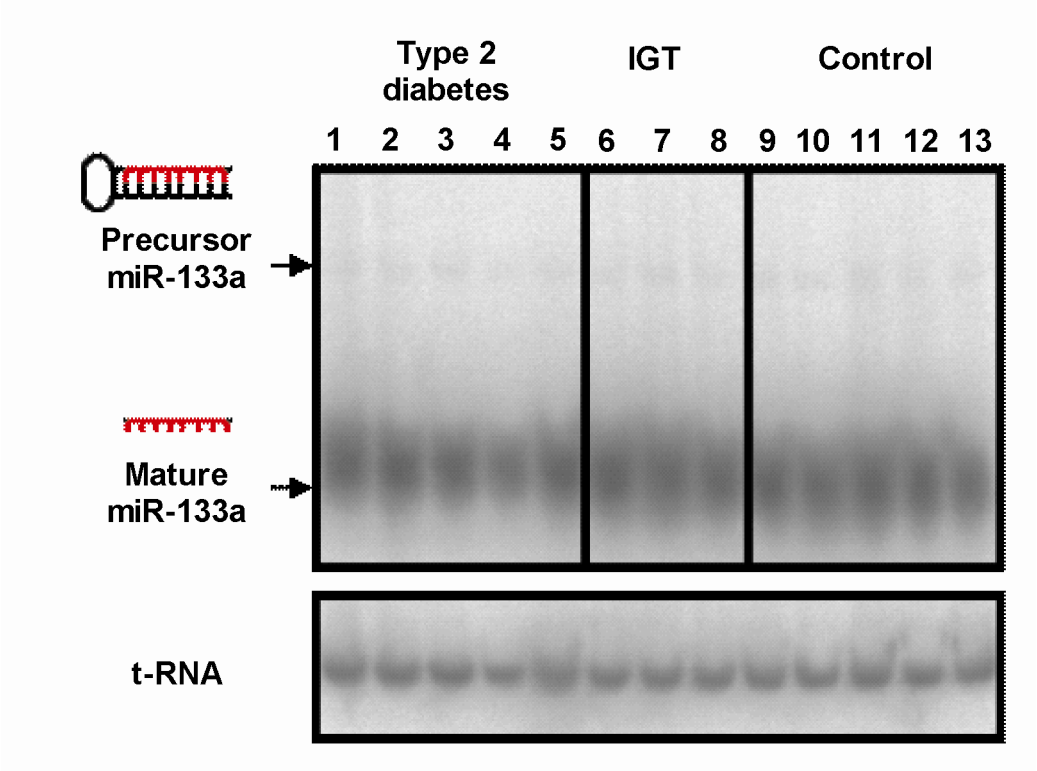


Figure 4-6. Northern blot of pre- and mature miR-133a expression. The miR-133a precursor is not strongly expressed, or is quickly processed into mature miR-133a. In comparison mature miR-133a is abundantly expressed in Type 2 diabetes, IGT and Control. Lanes 1-3, 6-8 and 6-11 are from pooled RNA. Lanes 4-5 and 12-13 are from individual patient's RNA.

4.3.4 Expression of microRNA processing genes in Type 2 diabetes

The lack of detectable changes in primary miR-133a transcription and the rapid processing of pre-miR-133a suggested some unknown factors are influencing the processing of pri-miR-133a-1 and/or pri-miR-133a-2. Therefore the expression of microRNA processing genes was examined for differences between the Type 2 diabetes, IGT and control group that may help explain why mature miR-133a and miR-206 were less abundant in Type 2 diabetes patients (Figure 4-7). There was no detectable decrease in genes encoding the nuclear primary microRNA processing proteins RNASEN (DROSHA) and DGCR8 in the Type 2 diabetes group compared to the IGT and control group (Figure 4-7). The cytoplasmic pre-microRNA processing proteins DICER1 and EIFC2C (AGO) were not detectably different between the Type 2 diabetes

and the control group (Figure 4-7). However, there was a significant difference in DICER1 detected between the IGT and the control group ($P < 0.01$).

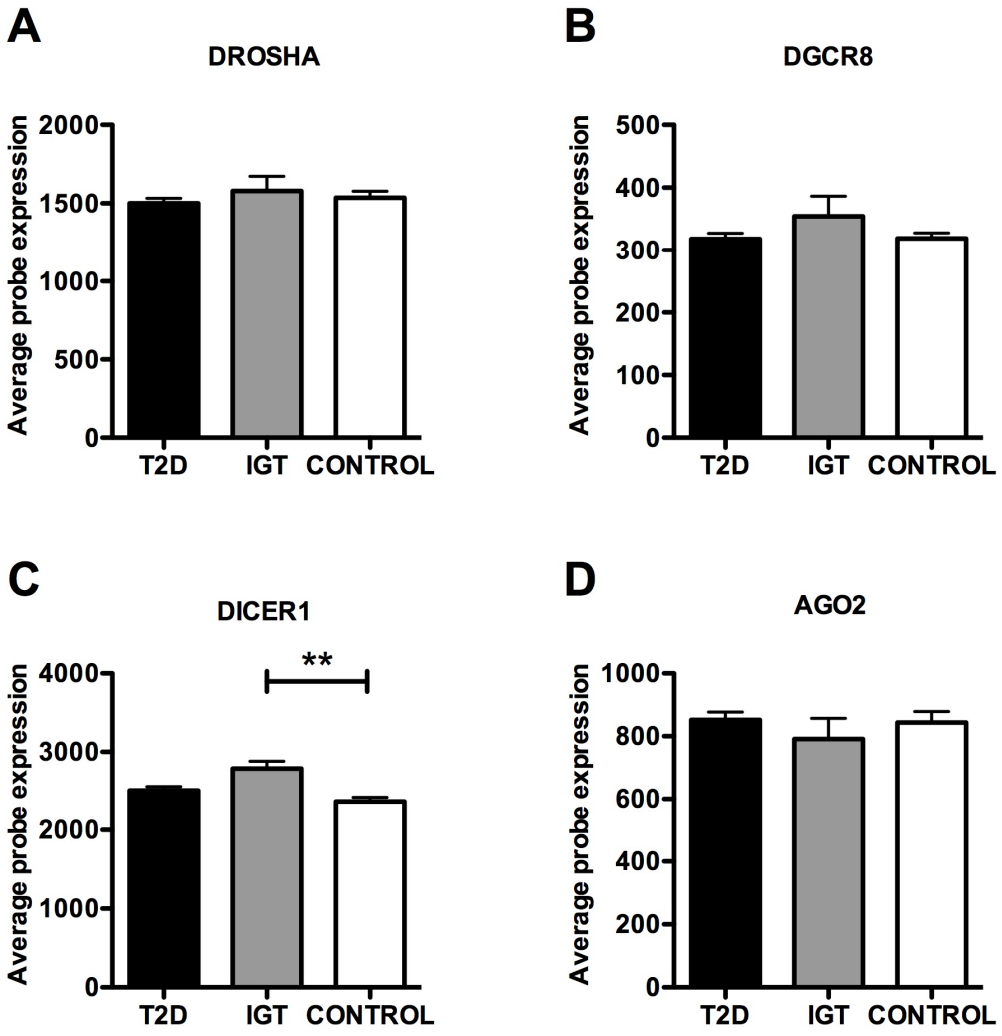


Figure 4-7. Expression of microRNA processing genes in Type 2 diabetes. (A) DROSHA, (B) DGCR8, (C) DICER1 and (D) AGO2 expression. Data is based on microarrays of skeletal muscle from Type 2 diabetes, IGT and control patients (n = 118). Data shown as mean ± SE. ** $P < 0.01$

4.3.5 Glucose homeostasis and insulin resistance predicted by miR-133a

Type 2 diabetes pathogenesis is associated with a progressive increase in insulin resistance, which puts stress on the pancreatic β-cells to secrete more insulin to compensate and maintain glucose homeostasis. Indicators of glucose homeostasis and insulin resistance are useful clinical markers of impaired glucose tolerance and Type 2 diabetes. If microRNAs can predict the decline in glucose tolerance they could represent novel biomarkers for the early detection of Type 2 diabetes. To answer this

question multiple regression was used to determine whether miR-133a or miR-206 could explain some of the variance in clinical indicators of insulin resistance and glucose homeostasis. Glucose tolerance, HbA1c, basal insulin and HOMA[IR] appeared not to be normally distributed (Supplementary Figure 4-6 and Supplementary Figure 4-7), and therefore were log transformed prior to multiple regression analysis. Fasting glucose was rank transformed prior to multiple regression analysis.

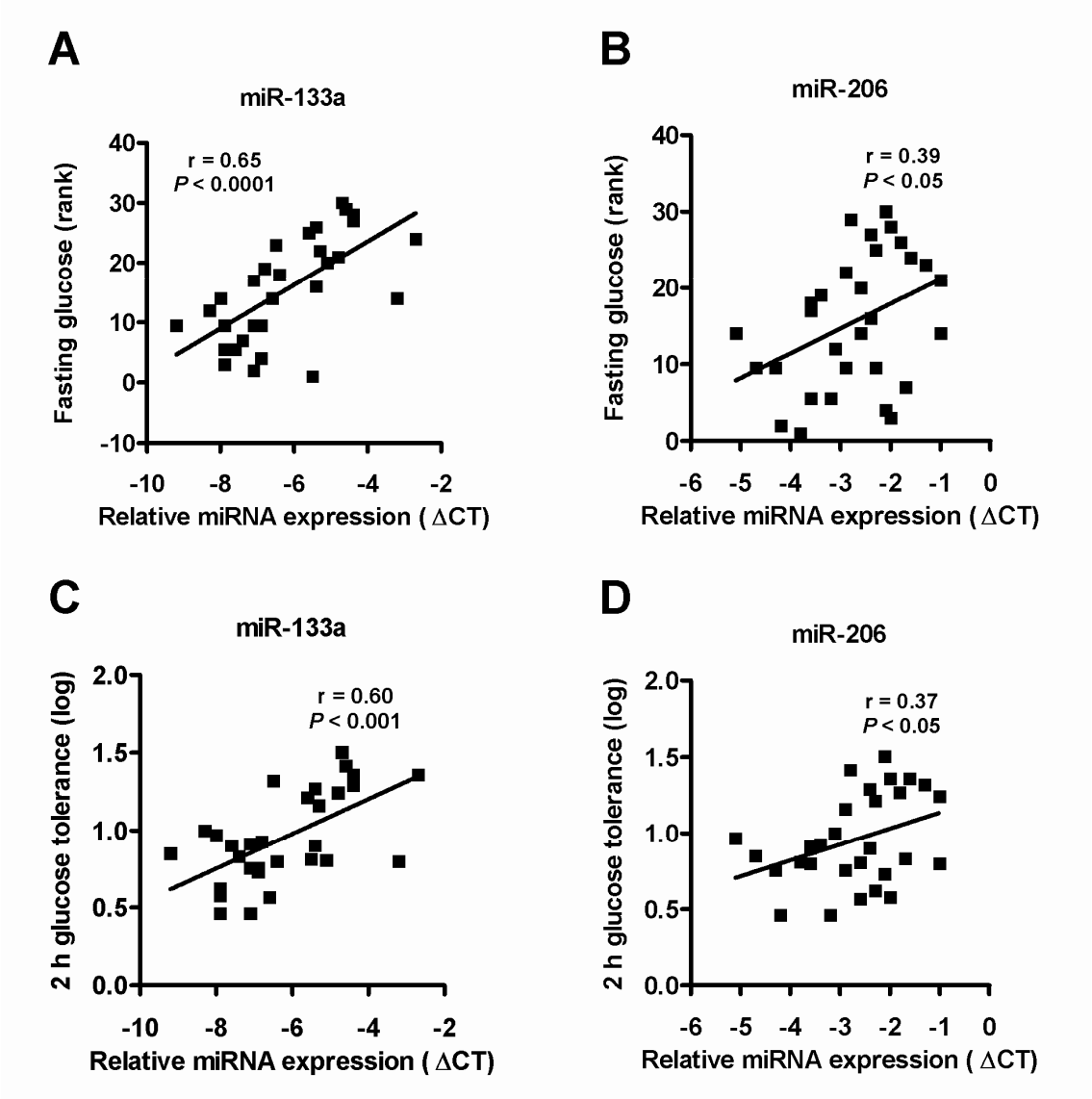


Figure 4-8. Association of miR-133a and miR-206 with (A and B) fasting glucose (rank transformed) (C and D) log 2 h glucose tolerance (n = 30).

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A multiple linear regression model including miR-133a and miR-206 explained 42% of the variance in fasting glucose ($F = 9.8$, $P < 0.001$). Residuals were randomly distributed (Supplementary Figure 4-5), there was no evidence of multicollinearity ($R^2 = 0.33$) and fasting glucose passed the KS normality test following rank transformation.

However, only miR-133a made a significant contribution to the model (Table 4-4), suggesting miR-133a could predict fasting glucose alone. A simpler regression model that included only miR-133a was found to explain 42% of the variance in fasting glucose concentrations (Figure 4-8; F = 20.4, $P < 0.0001$) based on the equation [Fasting glucose (mmol/L)] = 38.1 + 3.6*[miR-133a].

Table 4-4. Multiple regression model shows miR-133a predicts fasting glucose.

Variable	β coefficient	T ratio	*P* value
Constant	38.1 ± 5.3	7.2	<0.0001***
miR-133a	3.5 ± 1.0	3.5	0.001***
miR-206	0.1 ± 1.4	0.1	0.90

Based on rank transformed fasting glucose. *** $P < 0.001$ ** $P < 0.01$ ** $P < 0.01$

The oral glucose tolerance test provides a useful clinical indicator of patients' whole-body glucose disposal rate, which will slow down as insulin resistance develops. Multiple regression of miR-133a and miR-206 explained 36% of the variance in 2 h glucose tolerance (Figure 4-8; F = 7.6, $P = 0.002$) but miR-206 made no significant contribution to the model (Table 4-5). Residuals were randomly distributed (Supplementary Figure 4-5) and 2 h glucose tolerance passed the KS normality test following log transformation. However, a simpler regression model that included only miR-133a explained 36% of the variance in 2 h glucose tolerance (Figure 4-8; F = 15.6, $P < 0.001$) based on the regression model [2 h glucose tolerance] = 1.65 + 0.11*[miR-133a]

Table 4-5. Multiple regression model shows miR-133a predicts glucose tolerance.

Variable	β coefficient	T ratio	*P* value
Constant	1.65 ± 0.18	8.89	<0.0001***
miR-133a	0.11 ± 0.03	3.04	0.005**
miR-206	0.01 ± 0.05	0.23	0.81

Based on log transformed glucose tolerance data *** $P < 0.001$ ** $P < 0.01$ ** $P < 0.01$

HbA1c is an indicator of longer-term hyperglycaemia. HbA1c accumulates during prolonged hyperglycaemia when glucose binds to haemoglobin. Healthy HbA1c is below 7% and higher HbA1c is associated with increased susceptibility to Type 2

diabetes complications. A multiple regression model including miR-133a and miR-206 explained 31% of the variance in HbA1c (F = 6.0, P = 0.006), but only miR-133a made a significant contribution to the model (Table 4-6). Residuals were randomly distributed (Supplementary Figure 4-5) and HbA1c passed the KS normality test following log transformation (Figure 4-9). Although, a simpler regression model with only miR-133a predicted 30% of the variance in HbA1c (Figure 4-9); F = 12.3, P = 0.002) based on the regression model; [HbA1c] = 1.00 + 0.03*[miR-133a].

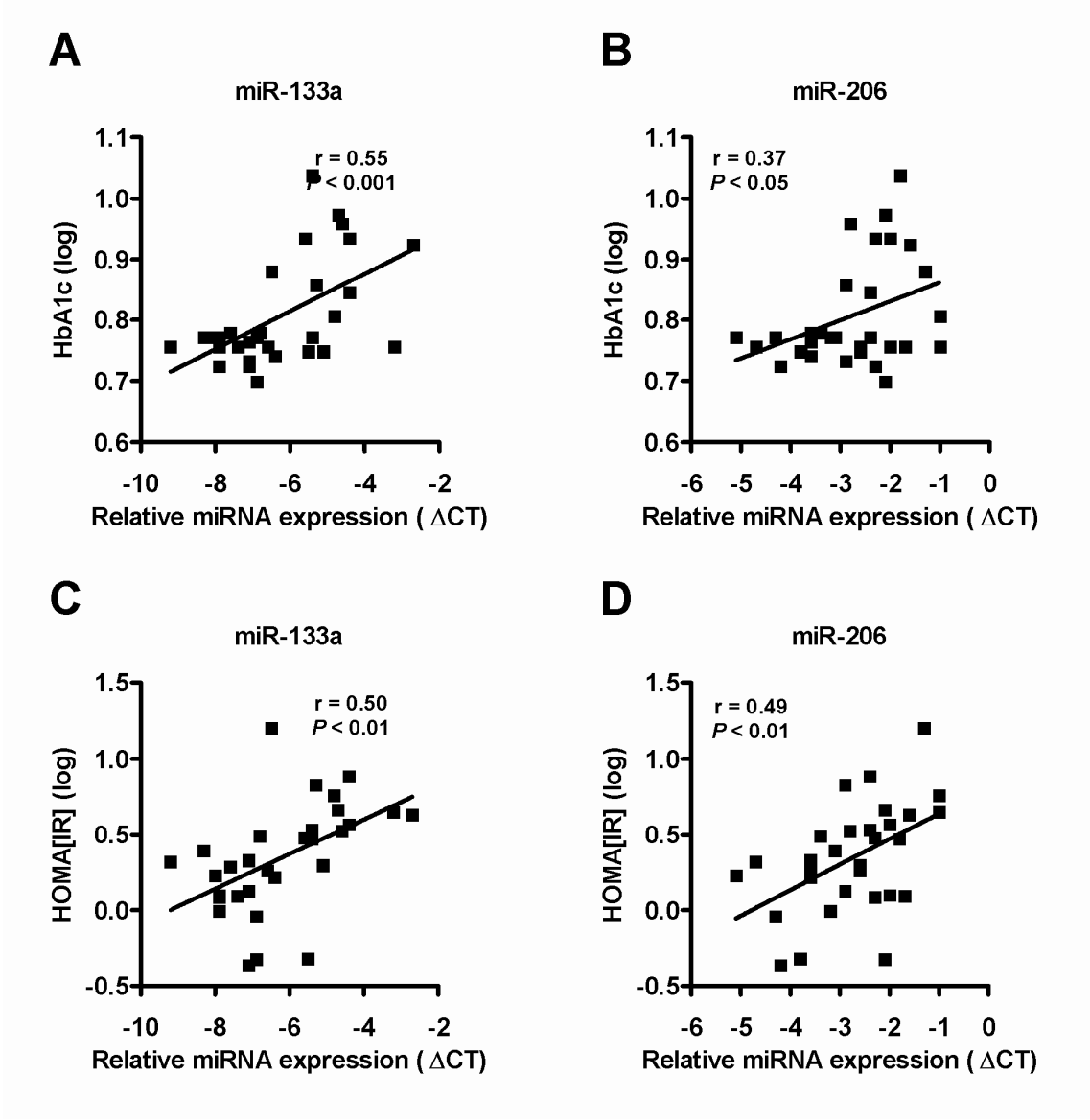


Figure 4-9. Association of miR-133a and miR-206 with (A and B) log HbA1c (C and D) HOMA[IR] (n = 30).

Table 4-6. Multiple regression model shows miR-133a predicts HbA1c.

Variable	β coefficient	T ratio	*P* value
Constant	1.00 ± 0.05	17.3	<0.0001***
miR-133a	0.02 ± 0.01	2.6	0.01**
miR-206	0.006 ± 0.01	0.4	0.68

Based on log transformed HbA1c data. *** $P < 0.001$ ** $P < 0.01$ ** $P < 0.01$

Basal insulin which is primarily determined by insulin secretion from pancreatic β-cells was not found to be correlated with skeletal muscle miR-133a or miR-206 expression (Supplementary Figure 4-7). However, HOMA[IR] which is an indicator of insulin resistance was predicted by a multiple regression model including miR-133a and miR-206 explained 31% of the variance in HOMA[IR] (Figure 4-9; F = 6.1, $P = 0.01$). HOMA[IR] passed the KS normality test after log transformation.

Table 4-7. Multiple regression model shows miR-133a and miR-206 can predict insulin resistance (HOMA[IR]).

Variable	β coefficient	T ratio	*P* value
Constant	2.00 ± 0.23	4.6	<0.0001***
miR-133a	0.07 ± 0.04	1.6	0.11
miR-206	0.10 ± 0.06	1.5	0.13

Based on log transformed HOMA[IR] data. *** $P < 0.001$ ** $P < 0.01$ ** $P < 0.01$

4.3.6 Overexpression of miR-133a targets diabetes associated genes

To demonstrate miR-133a may have a functional role in Type 2 diabetes it is important to experimentally validate miR-133a targets. Microarray data from HeLa cells 24 h following transfection of a miR-133a duplex designed to mimic endogenous miR-133a was retrieved from GEO (Grimson et al. 2007). Target expression was mapped to manually curated literature citations associated with diabetes. Forty-two miR-133a targets were identified with >1 diabetes associated citations in the NCBI database (Supplementary Figure 4-10). Based on the difference in log2 intensity between transfected and control cells the majority of diabetes linked miR-133a targets were suppressed. The largest decrease in a diabetes associated miR-133a target was for CDC42 which has been linked with GLUT4 trafficking (Nevins & Thurmond, 2005; Usui et al. 2003; Ishikura & Klip, 2008).

4.3.7 Evidence of microRNA action on target signatures in Type 2 diabetes

If miR-133a acts primarily through mRNA cleavage and degradation this would be reflected by an increase in miR-133a target abundance in Type 2 diabetes patients. In skeletal muscle, miR-1, miR-133a, miR-133b and miR-206 targets were significantly suppressed at mRNA level compared to miR-29a and miR-34a targets (Figure 4-10), which is in agreement with previous data showing microRNAs repress targets when highly expressed in a specific tissue.

However, there was no evidence of a decrease in miR-133a action on mRNA cleavage in Type 2 diabetes patients. The miR-133a target expression signature did not differ between patient groups (Figure 4-10), suggesting that miR-133a targets may be subject to translational repression rather than mRNA cleavage. Alternatively, the target expression signature is not sufficiently sensitive to detect differences in microRNA targets between the Type 2 diabetes, IGT and control groups.

The target expression signature of miR-1 and miR-206 was significantly increased in the Type 2 diabetes group compared to the control group (Figure 4-10; $P < 0.05$). Both miR-206 and miR-1 are from the same family, with similar seed sequences (Supplementary Table 4-2. miR-1/206 and miR-133 family sequence homology), and therefore share common targets. The reduced miR-206 target suppression was in agreement with the down-regulation of miR-206 observed in Type 2 diabetes patients (Figure 4-2).

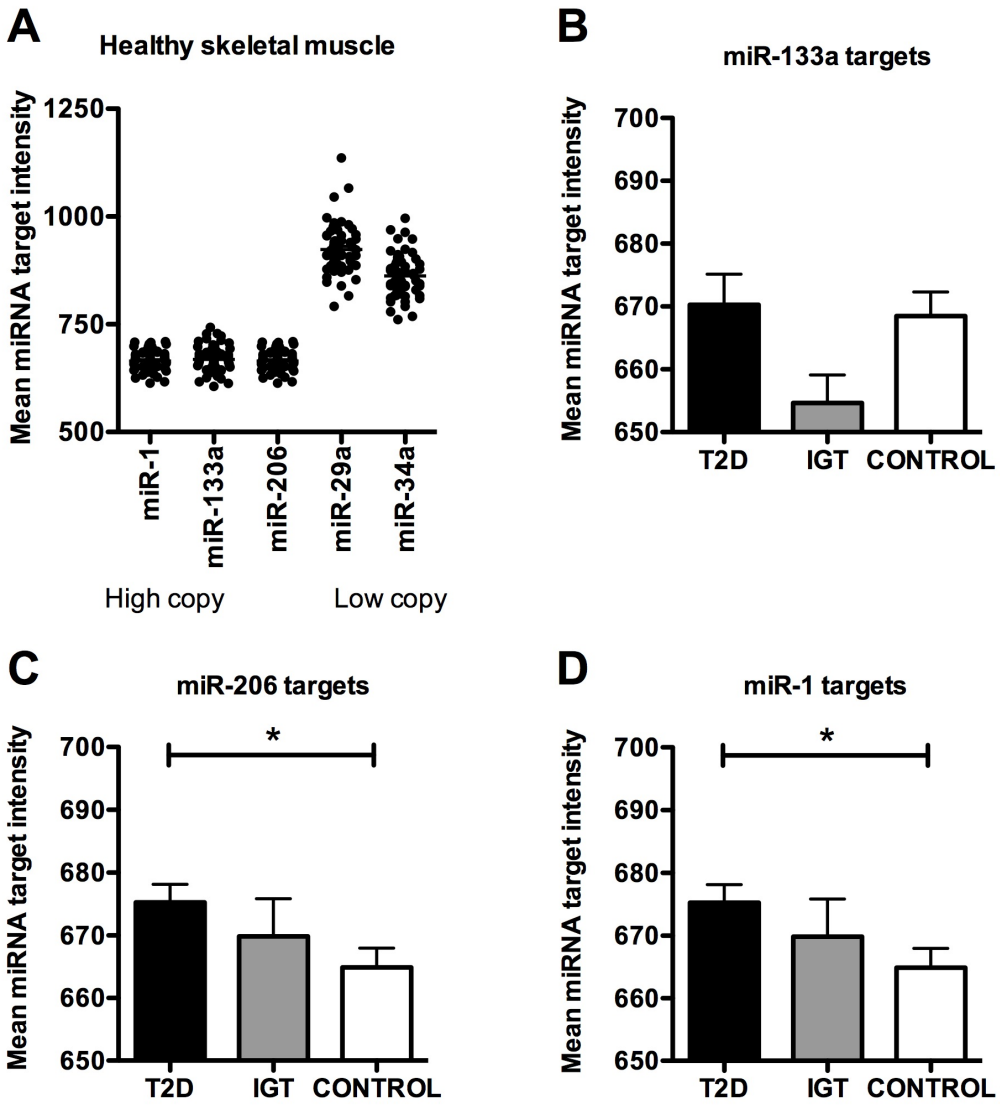


Figure 4-10. miR-1/miR-206 target expression signatures in Type 2 diabetes. (A) Target suppression by microRNAs with high copy number (miR-1, 206, 133a) compared to microRNAs with low copy number (miR-29a, miR-34a) in healthy skeletal muscle. Points represent mean microRNA target expression for individual patients. (B) miR-133a (C) miR-206 (D) miR-1 target expression in Type 2 diabetes, IGT and control patients. * $P < 0.05$

4.3.8 Gene ontology enrichment analysis

To examine the potential function of miR-133a in Type 2 diabetes, gene ontology enrichment analysis was conducted. Gene ontology (GO) enrichment analysis finds genes with similar biological function based on gene ontology terms as described in section 4.2.13. To limit the identification of spurious biological functions, only gene ontology term enrichment with a false discovery rate <10% were considered.

There was significant enrichment of GO terms associated with intracellular organelles and protein binding among miR-133a targets. Glucose uptake involves the intracellular trafficking of GLUT4 to the plasma membrane, which requires the intracellular organelle network (Bryant et al. 2002). The GO terms, Intracellular membrane-bound organelle, Clathrin-coated vesicle and Protein binding were significantly enriched with miR-133a targets. These included SVOP, AFTPH, AP2M1, CLTA, FURIN, VAT1, EGFR, LDLRAP1, SYT1 and VAMP2. It has been reported VAMP2 and VAMP3 are associated with SNARE interactions and vesicle transport (Bryant et al. 2002; Cheatham et al. 1996; Nevins & Thurmond, 2005), thus may be involved in GLUT4 trafficking. Furthermore, there was also significant enrichment of actin-associated GO terms including; Cytoskeletal protein binding, Actin cytoskeleton, Actin cytoskeleton organization and biogenesis. Reorganisation of the actin cytoskeleton may be required for movement of GLUT4 and insulin signaling proteins, although there have been few studies on the role of the actin cytoskeleton in Type 2 diabetes (Eyster & Olson, 2009).

Gene ontology terms linked to negative regulation of insulin signaling were also found. For example, miR-133a targets were significantly enriched with the GO term Protein phosphatase type 2A complex; miR-133a targets a group of protein phosphatase subunits including PPP2CB, PPP2R5D, PPP2R5E and PPP2R4. Protein phosphatase 2 (PPP2CB) inhibits insulin signaling via PI-3K (Begum & Ragolia, 1999). Impairment of PPP2CB down-regulation in response to insulin is reported in Type 2 diabetes patients (Højlund et al. 2002) and diabetic GK rats (Begum & Ragolia, 1999). Down-regulation of miR-133a is consistent with its potential action on protein phosphatase type 2A complex, which is a negative regulator of insulin signaling.

Finally, significant enrichment of GO terms linked to transcription were found, including Transcription from RNA polymerase II promoter, Transcription factor activity and Negative regulation of transcription, indicating that miR-133a targets transcription regulators and could have widespread indirect effects via regulation of transcriptional factor activity.

4.3.9 Identifying Type 2 diabetes biomarkers from microRNA target signatures

Patients' microRNA target expression signatures were analysed using Statistical Analysis of Microarrays (SAM) to identify further potential microRNA biomarkers in Type 2 diabetes. SAM provides control for multiple testing errors and calculates a false discovery rate for the differentially expressed microRNA target signatures. Based on a 0.02 FDR and a 1.8 delta cut-off, 248 microRNA target expression signatures were identified as differentially expressed between Type 2 diabetes and control patients which equates to ~15 false calls (Figure 4-11). Among these were the microRNA target expression signatures for miR-1 and miR-206 in agreement with the ANOVA results (section 4.3.7). The list of 248 microRNAs with differentially expressed target signatures is shown in Supplementary Table 4-4. However, the microRNA target signature for miR-133 was not identified using SAM. Further work should clarify whether microRNA target expression signatures correlate with microRNA expression.

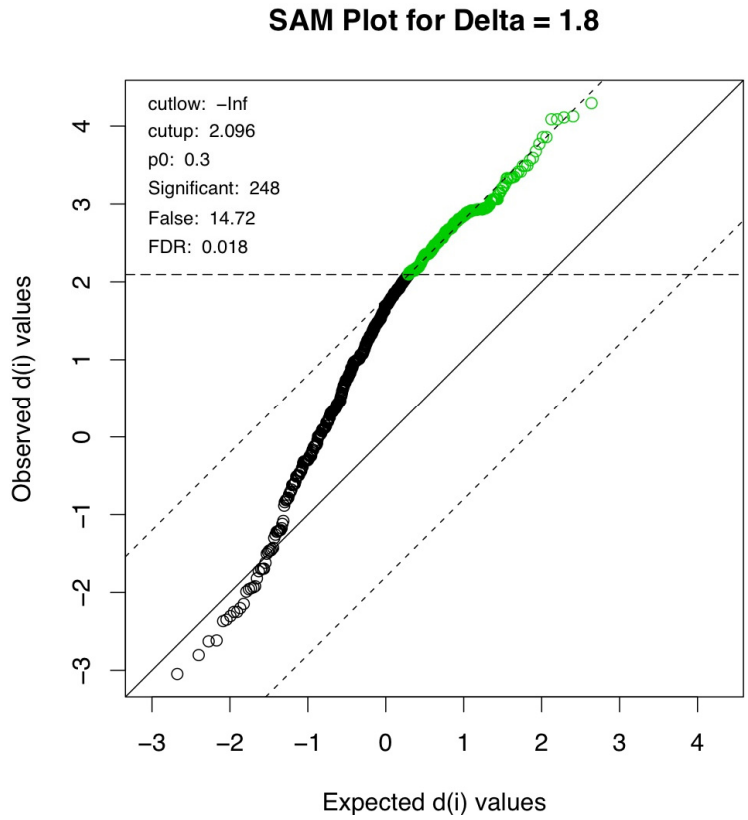


Figure 4-11. SAM analysis of microRNA target expression signatures may identify possible microRNA biomarkers of Type 2 diabetes. 248 microRNA target expression signatures were differentially expressed, with an estimates 15 false calls. Grey circles indicate differentially expressed microRNA target expression signatures in Type 2 diabetes patients.

4.3.10 Experimental validation of microRNA arrays

Experimental validation of microRNA arrays found no significant difference in miR-27b, miR-143, miR-208 or miR-424 expression between Type 2 diabetes, IGT and control groups (Figure 4-12). The fold change in miR-27b, miR-143, miR-208 and miR-424 expression detected from microRNA arrays on pooled RNA from Type 2 diabetes and control patients is shown in Supplementary Table 4-5. In the Type 2 diabetes group miR-143 appeared to be increased 200%, but variability in miR-143 expression within the Type 2 diabetes group may have contributed to the lack of significance (Figure 4-12).

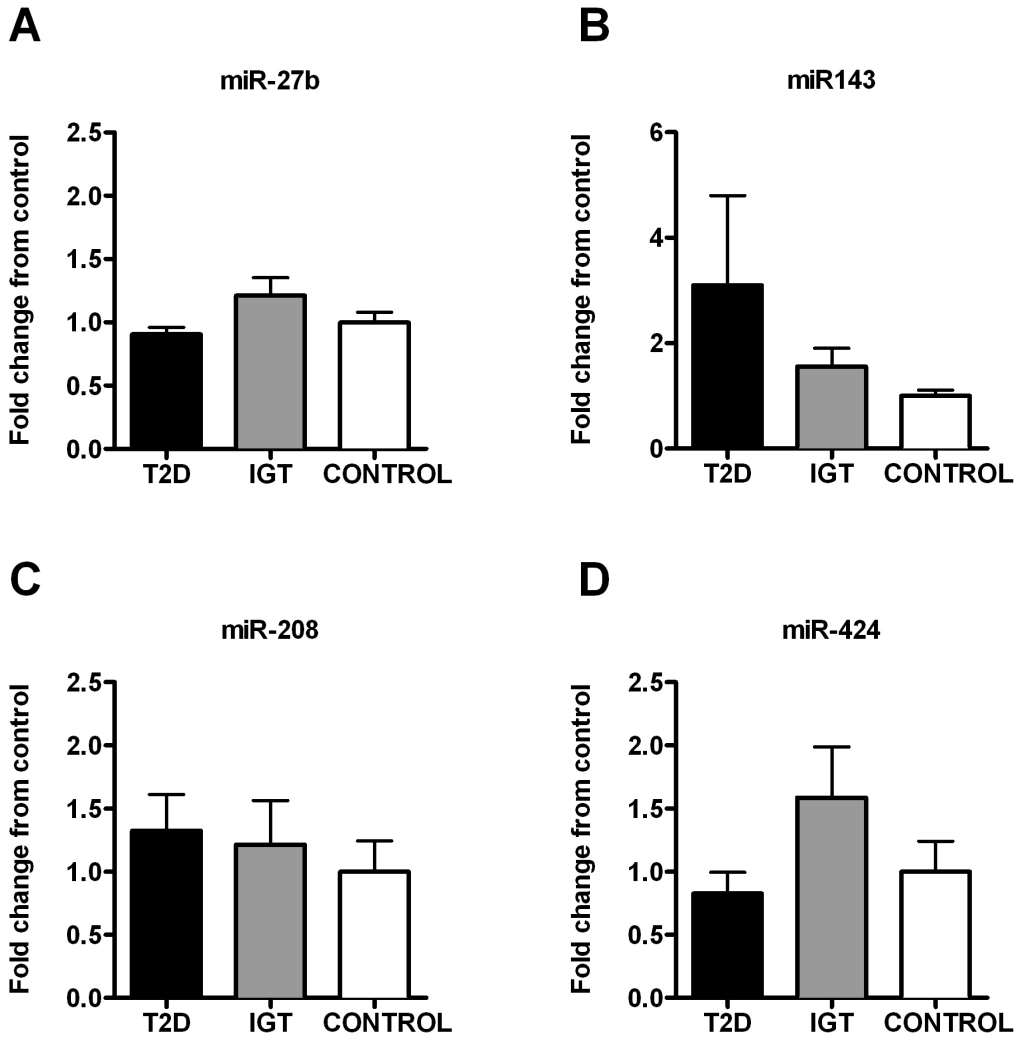


Figure 4-12. Fold change in (A) miR-27b, (B) miR-143, (C) miR-208 and (D) miR-424 expression in skeletal muscle from Type 2 diabetes, IGT and control patients. Data shown as mean ± SE.

The expression of miR-29a was significantly increased in the Type 2 diabetes compared to the control group ($P < 0.05$), but there was no significant difference in miR-29b expression between the Type 2 diabetes, IGT and control groups (Figure 4-13).

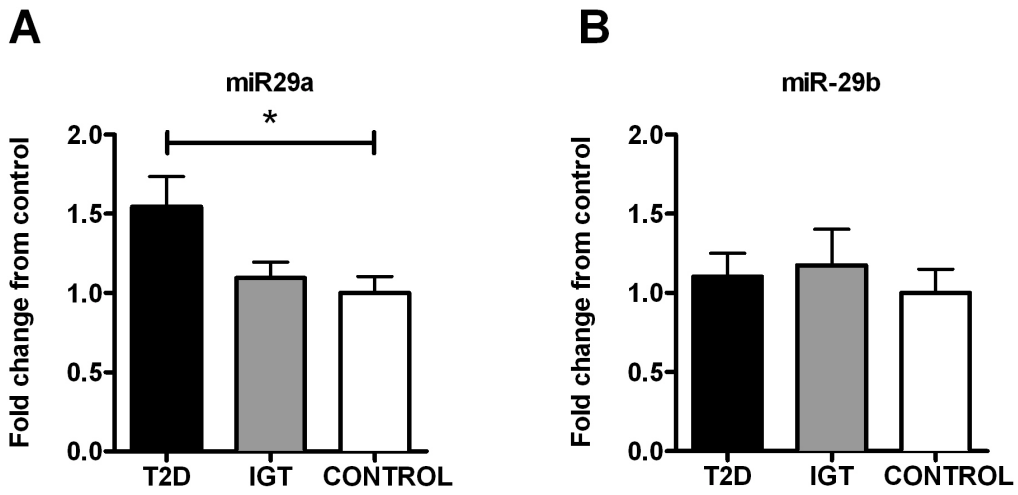


Figure 4-13. Fold change in (A) miR-29a and (B) miR-29b expression in skeletal muscle from patients with Type 2 diabetes, IGT and controls. Data shown as mean ± SE. * $P < 0.05$

Given that miR-29a expression appeared to be elevated in Type 2 diabetes, the next question was whether miR-29a had any detectable effect on target expression in Type 2 diabetes, IGT and control groups *in-vivo*. SAM analysis of all microRNA target expression signatures differentially expressed between the Type 2 diabetes and control group did not identify the miR-29a target expression signature (Supplementary Table 4-4). Furthermore, comparison of miR-29a target expression signature between the Type 2 diabetes, IGT and control groups showed no significant differences (Figure 4-14). The pathway enrichment analysis of miR-29a targets identified several pathways associated with Type 2 diabetes including insulin signaling, JAK/STAT, MAPK and regulation of the actin cytoskeleton (Figure 4-14).

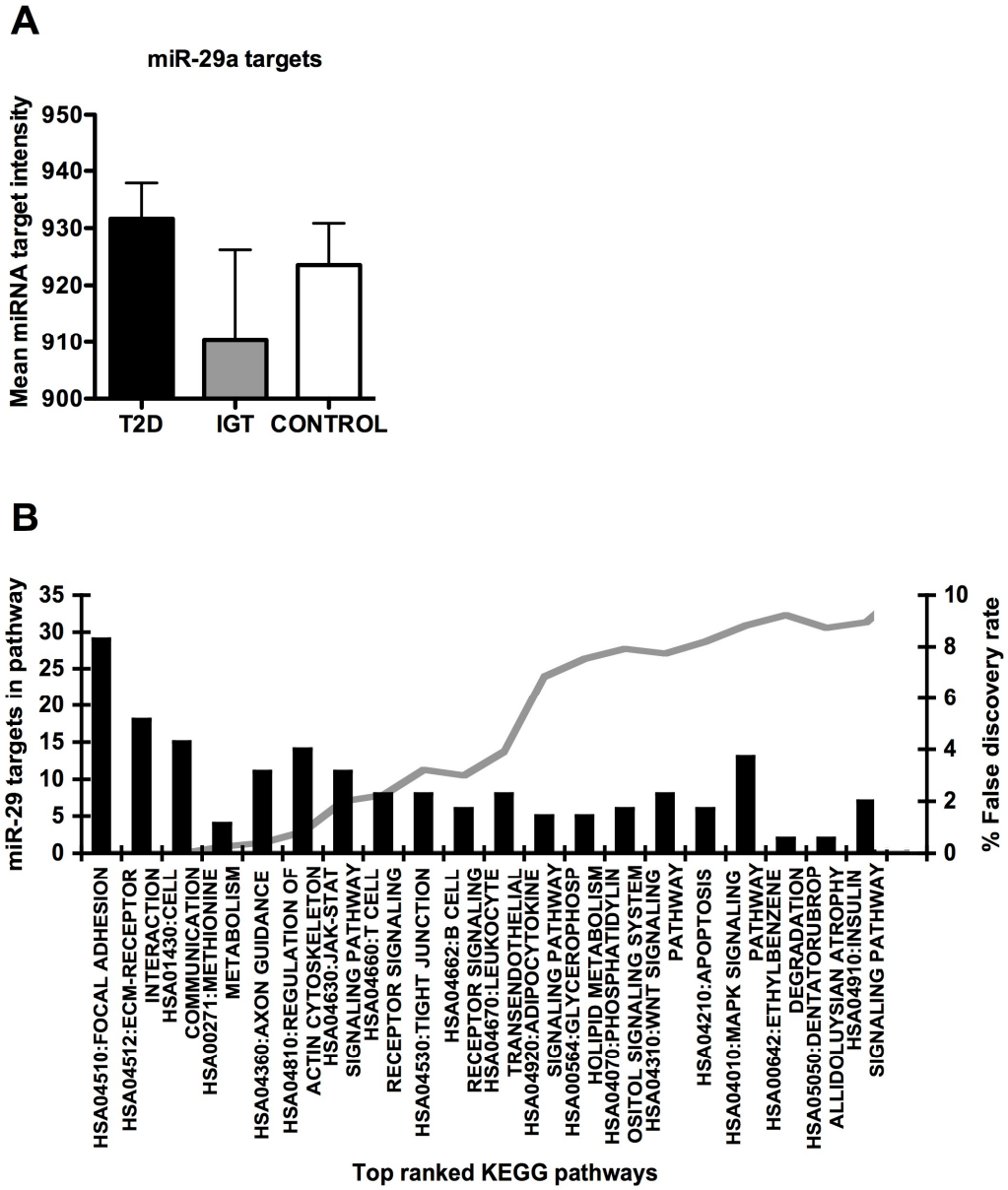


Figure 4-14. (A) No change in miR-29a target expression signature in Type 2 diabetes. (B) Top ranked KEGG Pathways enrichment with miR-29a targets.

4.4. Discussion

The main findings of this study were that in skeletal muscle from Type 2 diabetes patients, miR-133a and miR-206 expression are both significantly lower compared to IGT and controls, whereas miR-1 and miR-133b expression is similar. However, there was no change in miR-133a transcription and pre-miR-133a appeared to be rapidly processed, thus altered transcription appeared not to explain the changes in mature miR-133a in Type 2 diabetes.

Clinical indicators of glucose homeostasis and insulin resistance were predicted by miR-133a and miR-206 expression, but miR-133a appeared to be the strongest predictor. Furthermore, analysis of microarray data from HeLa cells after miR-133a overexpression showed miR-133a targets include diabetes-associated genes leading to mRNA cleavage. However, if miR-133a plays a role in skeletal muscle during Type 2 diabetes pathogenesis it appears miR-133a acts primarily via translation repression rather than mRNA cleavage, as no differences were found in miR-133a target expression in Type 2 diabetes patients. Interestingly, genes involved in protein phosphatase activity and intracellular transport were significantly enriched with predicted miR-133a binding sites, which suggests miR-133a could play a regulatory role in Type 2 diabetes pathogenesis. Alternatively miR-133a could be a promising candidate for use as a biomarker of dysregulated glucose homeostasis due to insulin resistance or pancreatic β-cell failure in Type 2 diabetes.

4.4.1 Down-regulation of miR-133a and miR-206 in Type 2 diabetes

The role of microRNAs during Type 2 diabetes development has not been studied previously in human skeletal muscle, although it is becoming clear that microRNAs play regulatory roles in numerous human diseases (Bushati & Cohen, 2007; Couzin, 2008; Perera & Ray, 2007). In the present study expression of two muscle-specific microRNAs, miR-133a and miR-206 was significantly lower in Type 2 diabetes patients compared to IGT and controls, which suggests they could play a regulatory role in Type 2 diabetes pathogenesis or alternatively may be a secondary effect in response to other physiological changes such as elevated glucose or insulin.

In diabetic GK rats with low glucose tolerance and Type 2 diabetes, miR-133a expression was not significantly different compared to healthy rats (He et al. 2007). Re-

analysis of the microRNA array data from that study using SAM with less stringent cut-offs still failed to identify miR-133a as differentially expressed (data not shown). This may have been due to limitations in the sensitivity of the microRNA array or may be due to fundamental differences in Type 2 diabetes pathogenesis between the diabetic rat model and human Type 2 diabetes.

Previous studies on the role of miR-133a in skeletal muscle development suggest that it is a regulator of proliferation (Chen et al. 2006). Overexpression of miR-133a in C2C12 myoblasts promotes proliferation and delays differentiation (Chen et al. 2006), although during skeletal muscle differentiation miR-133a expression is strongly induced together with miR-1 and miR-206 (Chen et al. 2006; Kim et al. 2006; Boutz et al. 2007). The role of miR-1, miR-133a and miR-206 in adult skeletal muscle is less clear and is unlikely to reflect only alterations in cell differentiation and proliferation. For example, miR-1, miR-133a and miR-206 were all found to be down-regulated in pancreatic cancer patients with cachexia (Chapter 3). However, miR-133a has also been reported to be down-regulated in a mouse skeletal muscle hypertrophy model (McCarthy & Esser, 2007). In addition, miR-133a is reported to be down-regulated in response to endurance exercise training in both mice and humans (Keller et al. 2007; Safdar et al. 2009). Furthermore, miR-133a has been shown to be down-regulated in regenerating zebrafish muscle (Yin et al. 2008). Taken together these studies suggest miR-133a and miR-206 may be down-regulated when tissue remodelling is required. Therefore, in Type 2 diabetes the decrease in skeletal muscle miR-133a and miR-206 expression may be a reflection of remodelling occurring.

Hyperglycaemia or hyperinsulinaemia may both contribute to modulating microRNA expression levels in Type 2 diabetes skeletal muscle, but there have been no studies examining microRNA expression in response to extracellular factors in human skeletal muscle. The stepwise decline in miR-133a expression between normal glucose tolerance, impaired glucose tolerance and Type 2 diabetes patients suggests miR-133a might be affected or may contribute to the development of hyperglycaemia or hyperinsulinaemia in Type 2 diabetes linked to insulin resistance and β-cell dysfunction. For example, in adipocytes it is reported miR-29 is responsive to extracellular glucose levels (He et al. 2007). In addition, miR-375 is responsive to extracellular glucose in pancreatic β-cells (El Ouaamari et al. 2008). Furthermore, a screen for glucose-regulated microRNAs identified 61 microRNAs out of 108 microRNAs were regulated

by glucose, but neither miR-133a nor miR-206 were detected in pancreatic β-cells (Tang et al. 2009). Taken together these studies suggest a screening approach could be useful in skeletal muscle to identify glucose-regulated microRNAs. It remains to be seen whether miR-133a or miR-206 expression is affected by extracellular glucose or insulin.

4.4.2 Transcription of miR-133a is unaltered in Type 2 diabetes

To determine whether the down-regulation of mature miR-133a in Type 2 diabetes was due to alteration in the transcription or processing of miR-133a, the expression of primary miR-133a transcripts and pre-miR-133a were measured. There was no evidence of an alteration in pri-miR-133a-1 or pri-miR-133a-2 expression in Type 2 diabetes or impaired glucose tolerance. The current literature assumes miR-1 and miR-133a are transcribed in unison from two independent bicistronic transcripts (Chen et al. 2006). In a mouse model of cardiac hypertrophy miR-1 and miR-133a expression are reported to change in a similar manner (Carè et al. 2007) supporting the assumption that they are transcribed together as a bicistronic transcript. The present findings suggest this may not be the case because mature miR-1 was unchanged while miR-133a was down-regulated in Type 2 diabetes, and pri-miR-1-2 appeared to be more abundant than its downstream neighbour pri-miR-133a-1 on chromosome 18. Several studies have reported discordant expression of mature miR-1 and miR-133a and their primary transcripts in agreement with the present data (Drummond et al. 2008; McCarthy et al. 2007). Taken together these findings indicate that changes in pri-microRNA transcripts do not always affect mature microRNA levels. Interestingly, pri-miR-133a-2 appears to be more abundant than pri-miR-133a-1, thus may be primarily responsible for determining mature miR-133a expression levels. In contrast, pri-miR-1-1 and pri-miR-1-2 appear to be expressed at similar absolute levels, suggesting they would contribute equally to determining mature miR-1 levels, but this could not be confirmed in the present study.

4.4.3 No evidence of altered processing of miR-133a in Type 2 diabetes

Pre-miR-133a expression indicated it must be rapidly processed into mature miR-133a, which is in agreement with a previous study indicating pre-miR-133a was present at very low levels in skeletal muscle (Lee et al. 2008). No differences in pre-miR-133a expression could be detected between Type 2 diabetes, impaired glucose tolerance or normal glucose tolerance suggesting pre-miR-133a processing was not responsible for

the down-regulation of mature miR-133a. In addition, the expression of microRNA processing proteins, DROSHA, DCGR8, AGO2 and DICER was similar between Type 2 diabetes, impaired glucose tolerance and normal glucose tolerance. Taken together these findings suggest there must be other regulatory steps after initial microRNA transcription that determines mature microRNA levels.

4.4.4 Glucose homeostasis and insulin resistance predicted by miR-133a

Multiple regression modelling was used to examine whether miR-133a and miR-206 expression were predictors of fasting glucose, basal insulin, HbA1c, HOMA[IR] or 2 h glucose tolerance. The relative expression of miR-133a and miR-206 was able to predict 30-40% of the variance in fasting glucose, HbA1c, 2 h glucose tolerance and HOMA[IR], but not basal insulin using the multiple regression approach. Further analysis using only miR-133a expression as a predictor of fasting glucose, HbA1c, 2 h glucose tolerance and HOMA[IR] found miR-133a alone was able to explain between 30-40% of the variance in these clinical measures of glucose homeostasis and insulin resistance. However, other factors also influence fasting glucose, HbA1c and 2 h glucose tolerance, which were not considered in this study. For example HbA1c, fasting glucose and 2 h glucose tolerance are strongly influenced by insulin secretion. However, miR-133a is not highly expressed in the pancreas and has not been linked to insulin secretion (Tang et al. 2009), which suggests that miR-133a most likely affects insulin resistance in Type 2 diabetes leading to secondary stress on β-cell function.

It is important to emphasise that the multiple regression models with miR-133a and miR-206 as predictors explained less than half of the variance in glucose homeostasis and insulin resistance indicators. The least squares regression approach assumes a linear relationship between microRNA expression and clinical variables, however fasting glucose, 2 h glucose tolerance, HbA1c, basal insulin and HOMA[IR] all showed greater variance in the Type 2 diabetes patients, although this was reduced by log transformation. Of the muscle-specific microRNAs profiled miR-133a appeared to be the best predictor of fasting glucose and 2 h glucose tolerance, which are both part of the current Type 2 diabetes diagnosis criteria and therefore could represent an early biomarker of Type 2 diabetes pathogenesis prior to β-cell failure. However, whether miR-133a expression could be a useful biomarker of impaired glucose tolerance and Type 2 diabetes would need to be established in a larger prospective study.

4.4.5 Potential regulatory role of miR-133a in Type 2 diabetes

MicroRNAs have been shown to play a regulatory role in diverse diseases, regulating differentiation, proliferation, apoptosis, and insulin secretion (Couzin, 2008). If miR-133a does play a regulatory role in Type 2 diabetes then examination of miR-133a target genes should reveal a group of genes that could contribute to the impaired glucose tolerance and insulin resistance in skeletal muscle.

The predicted targets of miR-133a were mined for significantly enriched gene ontologies using the bioinformatics functional classification tools available through NCBI/DAVID (Sherman et al. 2007). The calculation of enrichment was based on a modified Fischer test, which tests whether the microRNA target list contains a significant number of genes belonging to the same ontology group compared to the total number of genes in the human genome associated with the same ontology group. For example, genes involved in intracellular transport and protein dephosphorylation were significantly enriched among miR-133a targets. Enrichment of a gene ontology in a microRNA target list means that the microRNA may co-ordinately regulate a group of genes with similar functions (John et al. 2004). The data suggests the functional consequences of miR-133a in adult skeletal muscle may be partly through repression of intracellular transport proteins and protein phosphatases.

4.4.6 Protein phosphatases are predicted targets of miR-133a

In particular, genes associated with phosphoprotein phosphatase activity and membrane-bound organelles are of interest to Type 2 diabetes as these are involved in insulin-stimulated glucose uptake and hence have the potential to modulate glucose homeostasis. The miR-133a targets significantly enriched for phosphoprotein phosphatase activity and protein amino acid dephosphorylation ontologies included several protein-tyrosine phosphatases.

Protein-tyrosine phosphatases (PTPs) can reduce insulin receptor kinase activity by dephosphorylation of the insulin receptor (Youngren et al. 2007). *In-vivo* overexpression of PTP-1B in mice reduces insulin receptor autophosphorylation leading to insulin resistance (Zabolotny et al. 2004), whereas PTP-1B knockout mice have enhanced insulin receptor autophosphorylation and insulin sensitivity (Elchebly et al. 1999). In Type 2 diabetes patients, activity of PTPs appear to be elevated (Worm et al. 1996). The PTPs targeted by miR-133a include PTPN12, PTPRD, PTPRZ1 and protein

phosphatase subunits. Protein phosphatase 2 (PPP2CB) has been previously linked to Type 2 diabetes as it can inhibit insulin signaling through PI3K (Begum & Ragolia, 1999). In addition, PPP2CB down-regulation in response to insulin has been reported to be impaired in Type 2 diabetes patients (Højlund et al. 2002). Therefore, down-regulation of miR-133a in Type 2 diabetes may increase protein levels of protein phosphatases, which can impair insulin signaling.

In addition to identifying groups of genes that miR-133a may target co-ordinately in Type 2 diabetes, each target was examined independently for literature associations with diabetes. This revealed over 15 predicted miR-133a targets with previously established links to diabetes providing support for the regulatory role of miR-133a in Type 2 diabetes. However, only CDC42 has been previously experimentally validated as a miR-133a target in cardiomyocytes (Carè et al. 2007).

4.4.7 CDC42 and GLUT4 trafficking proteins are predicted targets of miR-133a

CDC42 is a small GTP binding protein that contains a highly conserved miR-133a target site. CDC42 has been confirmed as a miR-133a target based on a luciferase reporter assay in cardiomyocytes (Carè et al. 2007). The analysis of existing microarray data in HeLa cells from miR-133a transfections (Grimson et al. 2007) showed CDC42 and other genes down-regulated in response to miR-133a overexpression, thus providing further evidence supporting a regulatory role for miR-133a in Type 2 diabetes.

CDC42 can mediate insulin-stimulated GLUT4 transport and stimulate glucose uptake in 3T3-L1 adipocytes (Usui et al. 2003). In response to extracellular glucose CDC42 interacts with vesicle-associated membrane protein 2 (VAMP2) and relocates to the plasma membrane (Nevins & Thurmond, 2005). VAMP2 contains a predicted miR-133a binding site, therefore could also be regulated by miR-133a. Furthermore, diabetes-associated miR-133a targets included RAB14, which is a substrate of the Rab-GTPase activating protein AS160 and insulin-stimulated GLUT4 translocation is reported to require AS160 phosphorylation (Larance et al. 2005). In addition, knockdown of RAB14 in muscle cells has been shown to inhibit insulin-induced GLUT4 translocation (Ishikura & Klip, 2008). Taken together these studies suggest miR-133a may co-ordinately target GLUT4 trafficking proteins and therefore help regulate insulin-stimulated glucose uptake.

4.4.8 SOCS proteins are predicted targets of miR-133a

The suppressor of cytokine signaling (SOCS) was also found to be a miR-133a target previously associated with Type 2 diabetes. It has already been reported SOCS proteins can be regulated post-transcriptionally by microRNAs (Pichiorri et al. 2008). For example, in multiple myeloma the miR-19 family was shown to target SOCS-1, which is frequently silenced in multiple myeloma (Pichiorri et al. 2008). The re-analysis of microarray data in HeLa cells following overexpression of miR-133a showed SOCS might be regulated mainly through translational repression.

The SOCS family act as negative regulators of JAK/STAT mediated cytokine signaling (Balasubramanyam et al. 2005). SOCS proteins have distinct binding sites for IRS and elongin BC ubiquitin-ligase (Rui et al. 2002). Binding of SOCS1 and 3 to the insulin receptor can lead to degradation of insulin receptor substrates, IRS-1 and IRS-2 (Balasubramanyam et al. 2005). Thus SOCS proteins can suppress insulin signaling downstream of IRS, although the action of miR-133a on SOCS in human skeletal muscle has not yet been confirmed.

4.4.9 MicroRNA target signatures reveal novel Type 2 diabetes microRNAs

It has been shown that microRNA target signatures can be detected in microarray data from different tissues (Sood et al. 2006). Furthermore, microRNA target signatures in tissues can be detected based on the mean absolute expression of microRNA targets (Arora & Simpson, 2008). Thus existing microarray datasets could be mined for microRNA target signatures, which is potentially useful for identifying novel candidate microRNAs (Arora & Simpson, 2008).

SAM analysis identified the microRNA target signature of miR-206 as significantly different in the Type 2 diabetes compared to the control group. However, the microRNA target signatures of miR-133a and miR-29a were not found to be significantly different between Type 2 diabetes and controls, which suggests that using SAM analysis to find differentially expressed microRNA target signatures between disease groups requires further optimisation. For example, microRNA target signatures could be limited to microRNA targets with 8mer site matches only, as 8mer sites are suggested to be more effective at target suppression than 7mer-8 and 7mer sites (Grimson et al. 2007; Baek et al. 2008; Bartel, 2009). There are currently no examples of microRNAs where all targets have been experimentally validated. Therefore, it is

difficult to directly test the utility of using microRNA target signatures to identify possible disease linked microRNAs.

It will be important to experimentally validate miR-133a targets potentially associated with Type 2 diabetes or insulin resistance in a muscle cell model, as this will provide further evidence to show whether miR-133a is a post-transcriptional regulater of insulin resistance and Type 2 diabetes. It remains to be seen whether microarray data can be mined for disease specific microRNA signatures based on comparing the expression of a group of microRNA targets for diseased and healthy tissue. Until then microRNA arrays remain the only way to identify many potential disease biomarkers simultaneously.

4.4.10 MicroRNA array validation confirms miR-29a up-regulation

The experimental validation of microRNA array using RT-qPCR confirmed miR-29a was up-regulated in Type 2 diabetes patients, which was in agreement with microRNA array and Northern data from GK diabetic rats showing miR-29a up-regulation in adipose and muscle tissue (He et al. 2007). However, in contrast despite being identified as differentially expressed based on microRNA arrays, miR-27b, miR-29b, miR-143, miR-208 and miR-424 were not found to be differentially expressed using RT-qPCR. It is difficult to explain the lack of agreement between the microRNA array data and the RT-qPCR validation data presented here as the microRNA arrays were conducted independently (I. Gallagher, personal communication). However, pooling patient RNA for the microRNA arrays is likely to have obscured some of the individual variance in microRNA expression preserved during the RT-qPCR validation. In the case of miR-143, variation in miR-143 expression in the Type 2 diabetes patients most likely explains the lack of significant differences between Type 2 diabetes patients and controls. Therefore, in future studies microRNA arrays should ideally be conducted on individual patients rather than pooled patient RNA.

Comparison of the present results from human Type 2 diabetes with microRNA array data from GK rats reveals little concordance between studies. For example recent microRNA array data from diabetic GK rats failed to show miR-29a was over expressed in Type 2 diabetes skeletal muscle (Huang et al. 2009). In this study miR-24, miR-126, miR-424, miR-23a, miR-450 and miR-130 were reported to be >2-fold down regulated, while miR-301 and let-7f were over 2-fold up-regulated (Huang et al. 2009). Previously

in diabetic GK rats miR-29a, miR-29b, miR-29c and miR-150 were reported to be up-regulated (He et al. 2007), while miR-379, miR-127, miR-299, miR-434, miR-335, miR-130, miR-19b, miR-451, miR-148, miR-199 and miR-152 were reported to be down-regulated (He et al. 2007). Therefore, care should be taken when interpreting microRNA studies based on murine Type 2 diabetes models, as they may not represent human Type 2 diabetes.

The lack of concordance between two microRNA array studies conducted in diabetic GK mice may have been due to the use of different custom microarray platforms (Huang et al. 2009; He et al. 2007). A recent study compared five commercial microRNA array platforms for agreement and reported a lack of agreement between some platforms (Sato et al. 2009). The finding that miR-29a was up-regulated in Type 2 diabetes following microRNA array validation work using RT-qPCR suggests it may be worth following up in a future study to determine its role in Type 2 diabetes. Knockdown of miR-29 in adipocytes suggests it may influence insulin-stimulated glucose uptake via impairment of Akt phosphorylation, but this has not been confirmed in skeletal muscle (He et al. 2007). Therefore, miR-29a appears to have the potential to modulate glucose homeostasis in Type 2 diabetes.

4.4.11 Limitations

Evaluating the predictive power of individual microRNA expression to explain clinical indicators of glucose tolerance and insulin resistance is reliant on accurate diagnosis. Ideally, a hyperinsulinaemic-euglycaemic clamp should be used, but this was not practical in the present study. Glucose tolerance is a dynamic response, which will be susceptible to biological variation. Even using the gold standard hyperinsulinemic clamp the within subject coefficient of variation (CV) is reported to be around 12%. To minimise biological variation the oral glucose tolerance test was conducted at the same time of day for all patients, although the WHO recommends two independent tests before any diagnosis to improve reliability (WHO, 2006).

The clinical diagnosis of impaired glucose tolerance and Type 2 diabetes is categorical based on oral glucose tolerance, but glycaemia control is a continuous variable thus patients close to clinical cut-off criteria may be incorrectly classified. This was highlighted a decade ago when the WHO followed the ADA and lowered the glycaemic threshold for identifying impaired glucose tolerance (WHO, 2006; Shaw et al. 2000).

Nevertheless, it is important that any microRNA expression profile can distinguish between IGT and healthy patients, because it is well established that IGT is a risk factor for cardiovascular disease and a predictor of all cause mortality (Balkau et al. 1999; Brunner et al. 2006; DECODE STUDY GROUP, 1999; Saydah et al. 2001).

Comparing patients' microRNA or microRNA target signatures is dependent on well-defined patient groups with similar characteristics, and while every effort was made to control for known confounders such as age, BMI and VO_2max, the IGT group included individuals with varying levels of glucose tolerance which would influence the power of any predictive model based on microRNA expression. Furthermore, although miR-133a predicted around ~40% of variation in glucose homeostasis indicators, a single microRNA is unlikely to explain the complex disease process.

Both miR-133a and miR-206 are predicted to target between 200-300 genes. The identification of microRNA targets is important in determining the function of these small molecules in healthy and insulin resistant muscle. There are an ever-growing number of prediction algorithms for determining functional microRNA:mRNA target interactions, for example TargetScan, PicTar and Miranda (Lall et al. 2006; John et al. 2004; Lewis et al. 2003). These algorithms vary in the rules they apply to determine functional targets (Sethupathy et al. 2006). For the analysis of miR-133a targets the TargetScan algorithm was used. This has been shown to be more sensitive than many other algorithms, meaning it can identify ~60% of functional targets (Sethupathy et al. 2006). However, potentially important non-conserved sites will not be considered when using TargetScan, as it relies on conservation of microRNA:mRNA interactions across species. During preparation of this thesis TargetScan was updated and now includes non-conserved microRNA target predictions (Friedman et al. 2009). It is uncertain how effective non-conserved microRNA target sites are in post-transcriptional repression, as they have not been widely studied. Another issue is most algorithms provide global microRNA target lists, thus targets could be ranked as important even when microRNA or mRNA are not expressed in a tissue.

In the future a global profiling approach to identify insulin resistance and Type 2 diabetes associated microRNAs, mRNA and proteins would be useful, as this has proved successful in other chronic diseases such as cancer. For example unique microRNA signatures have been identified in lung cancer, which can predict patient

survival and relapse. There are limitations in using microRNA signatures to identify regulatory microRNAs in Type 2 diabetes because there are no simultaneous mRNA and microRNA profiles available for Type 2 diabetes. Therefore, the effectiveness of microRNA signatures in identifying relevant microRNAs could not be evaluated and can only be used as a guide to potential microRNAs, which may be dysregulated in disease. Finally the identification of a disease microRNA signature does not confirm whether microRNA dysregulation is the main cause of disease or only a biomarker of disease pathogenesis.

4.4.12 Future Directions

Global microRNA profiling in Type 2 diabetes will undoubtedly provide a new insight into post-transcriptional regulation in Type 2 diabetes pathogenesis. This is an especially powerful approach to use when combined with global mRNA and proteomic profiling (Baek et al. 2008; Selbach et al. 2008). However, the range of proteins detectable using current methods is limited compared to coverage of the human genome on Affymetrix microarrays. Based on the findings from the present study with the time and resources currently available it was important to identify and experimentally validate miR-133a and miR-206 targets (Chapter 5), which may contribute to Type 2 diabetes pathogenesis. This should provide additional evidence to show microRNAs are potentially influential post-transcriptional regulators in Type 2 diabetes pathogenesis. This will require a muscle-cell model, which can be transfected with microRNA knockdown oligonucleotides. Western analysis can be used to identify any shift in target protein expression following microRNA knockdown.

In addition, to establish whether these muscle-specific microRNAs are possible regulators of insulin resistance another patient cohort characterised by insulin resistance could be used such as aging, physical inactivity or obesity. Identifying post-transcriptional regulators of skeletal muscle phenotype in Type 2 diabetes is important given the central role of muscle insulin resistance in Type 2 diabetes pathogenesis (Mootha et al. 2003; Muoio & Newgard, 2008). In addition identification of novel microRNA biomarkers could eventually help facilitate earlier diagnosis of Type 2 diabetes, which would significantly improve morbidity and mortality rates (Roglic et al. 2005; Shaw et al. 2000; Wild et al. 2004; WHO, 2006; Zimmet et al. 2001). Only small amounts of RNA are required for microRNA analysis by RT-qPCR, which in addition is relatively quick compared to other methods such as Northern blot (Cissell & Deo, 2009;

Chen et al. 2005). It would be particularly useful to determine whether microRNA changes in serum correspond to microRNA changes in skeletal muscle, as a blood based biomarker would be more practical for clinical use.

4.4.13 Conclusions

- Muscle-specific miR-133a and miR-206 are down-regulated in Type 2 diabetes. Fasting blood glucose and 2 h glucose tolerance, part of the current Type 2 diabetes diagnostic criteria (WHO, 2006) can be partly predicted from miR-133a expression.
- Transcription of miR-133a is unaltered in Type 2 diabetes, there appears to be a post-transcription blockage in miR-133a processing. However, the expression of microRNA biogenesis proteins is unaltered in Type 2 diabetes.
- MicroRNA-133a is predicted to target diabetes associated genes including protein tyrosine phosphatases and vesicle transport proteins.
- MicroRNA target signatures can be used to mine existing microarray data for novel candidate Type 2 diabetes microRNAs.
- MicroRNA-29a was found to be up-regulated in human Type 2 diabetes in agreement with studies in diabetic rats.

4.5. Supplementary Data

Supplementary Table 4-1. Patient plasma inflammatory marker concentrations.

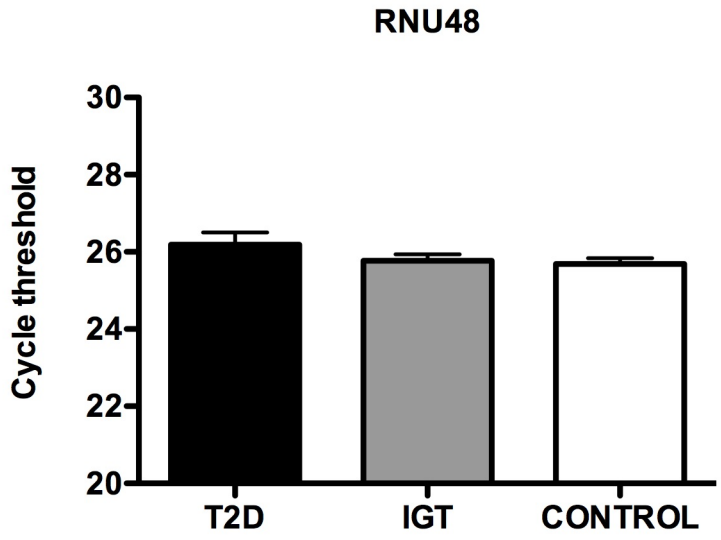
Patient characteristics	**Type 2 diabetes (n = 10)**	**IGT (n = 10)**	**Control (n = 10)**
CRP (mg/l)	2.2 ± 1.9	7.2 ± 11.8	1.3 ± 0.5
IL-6 (ng/l)	2.4 ± 3.4	2.4 ± 4.8	1.0 ± 0.5
IL-15 (ng/l)	1.5 ± 0.4	1.6 ± 0.4	1.8 ± 0.5
IL-18 (ng/l)	306 ± 121	339 ± 196	232 ± 74
TNFα (ng/l)	2.6 ± 0.5	2.2 ± 0.8	2.4 ± 0.6
TNFR2 (ng/l)	203 ± 18	201 ± 42	171 ± 35

Data are presented as mean ± SD.

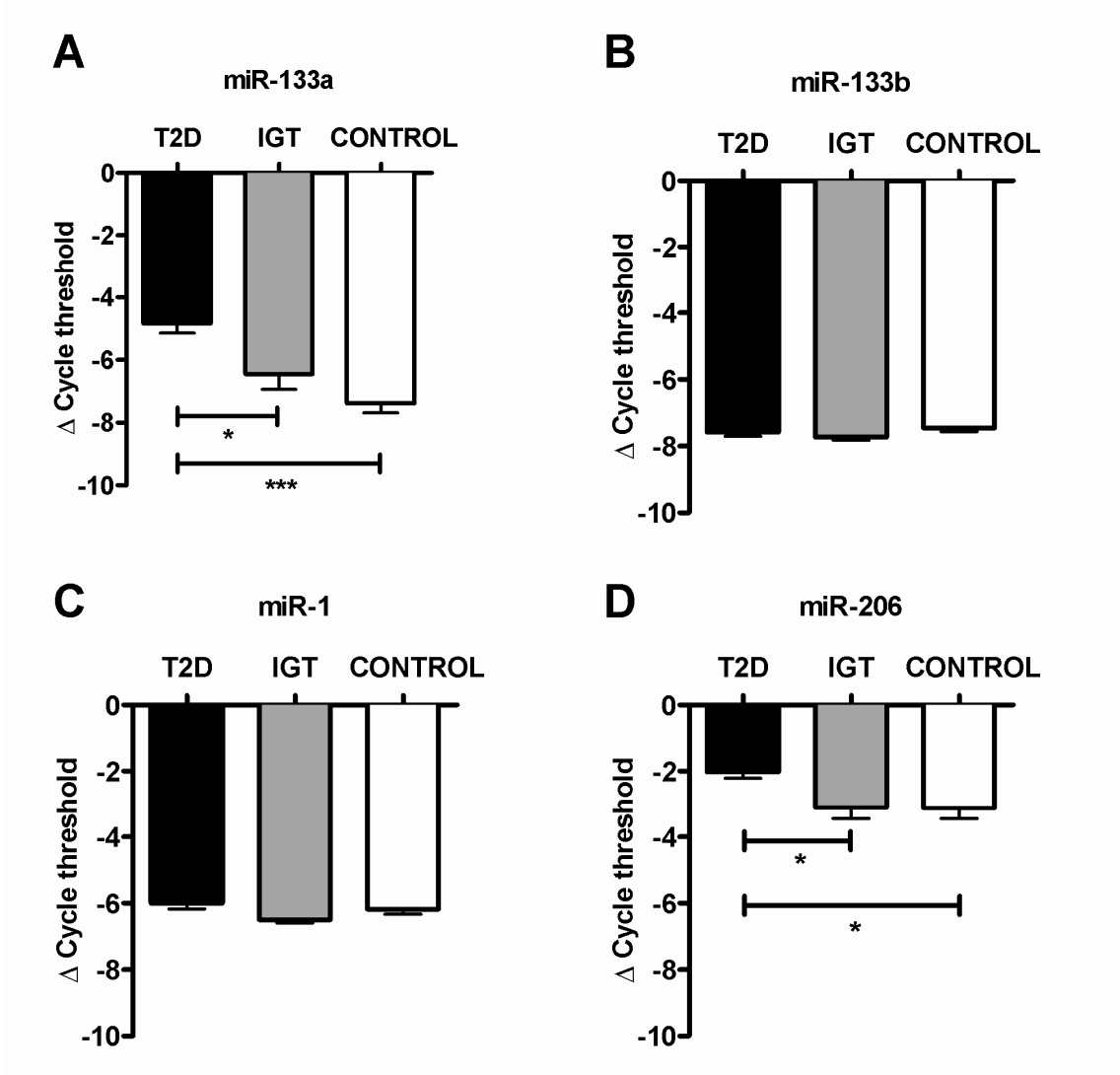
* $P < 0.05$ when compared with the control group.

** $P < 0.01$ when compared with the control group.

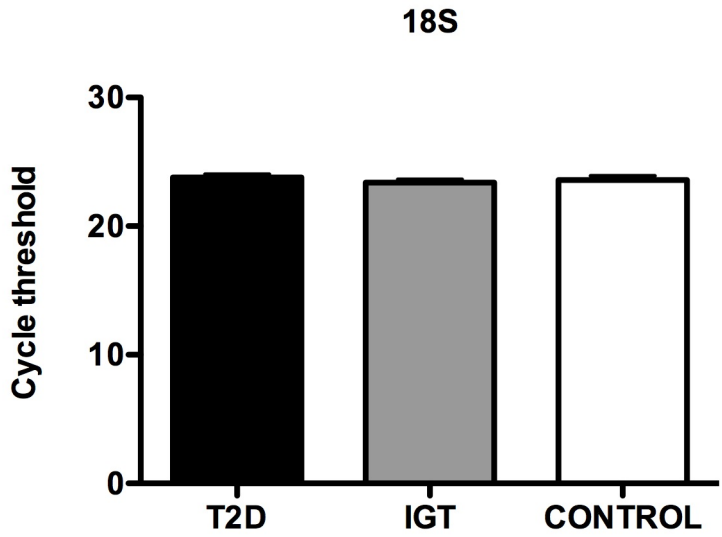
*** $P < 0.0001$ when compared with the control group.



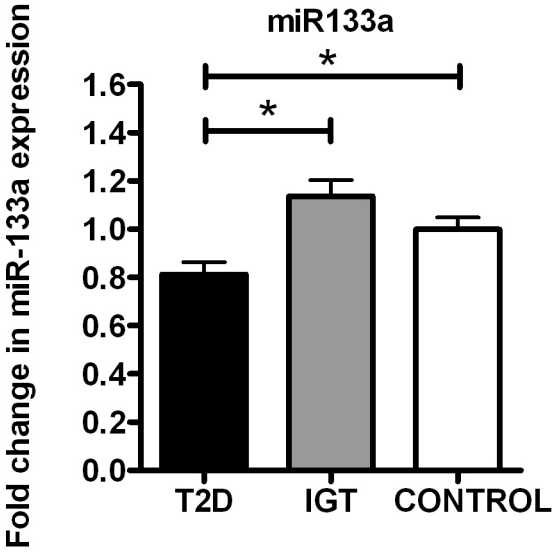
Supplementary Figure 4-1. RNU48 was stably expressed in Type 2 diabetes, IGT and controls. RNU48 was used as the housekeeping gene to normalise mature microRNA expression levels.



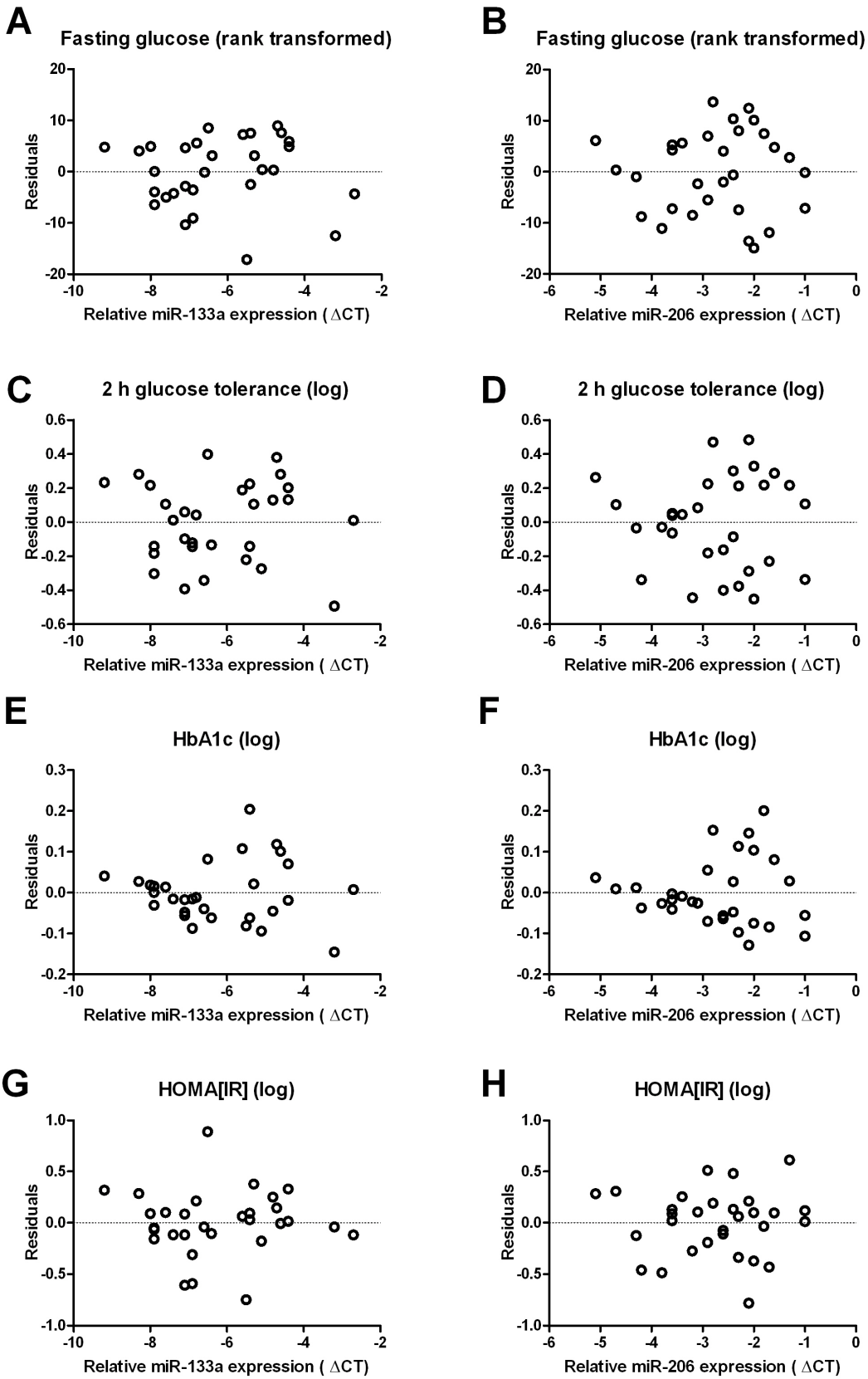
Supplementary Figure 4-2. ΔCycle thresholds for mature miR-133a, miR-206, miR-1 and miR-133b in the Type 2 diabetes, IGT and control group. A lower microRNA abundance corresponds to a higher cycle threshold. A higher microRNA abundance corresponds to a lower cycle threshold, miR-206 appeared to be less abundant than miR-1, miR-133a and miR-133b. *** $P < 0.001$ * $P < 0.05$



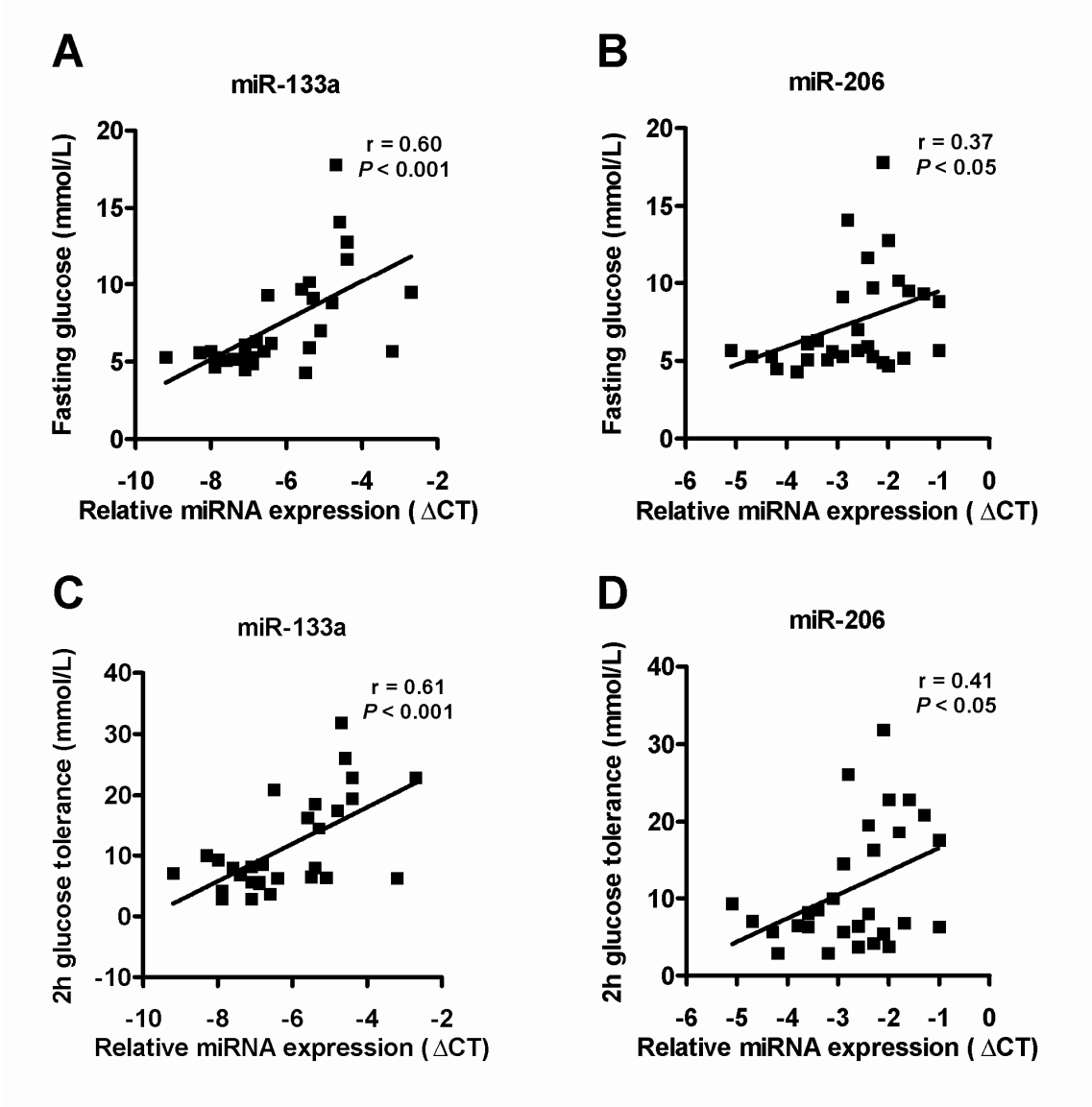
Supplementary Figure 4-3. 18S was stably expressed in Type 2 diabetes, IGT and controls. 18S was used to normalise pri-microRNA expression. Data shown as mean ± SE.



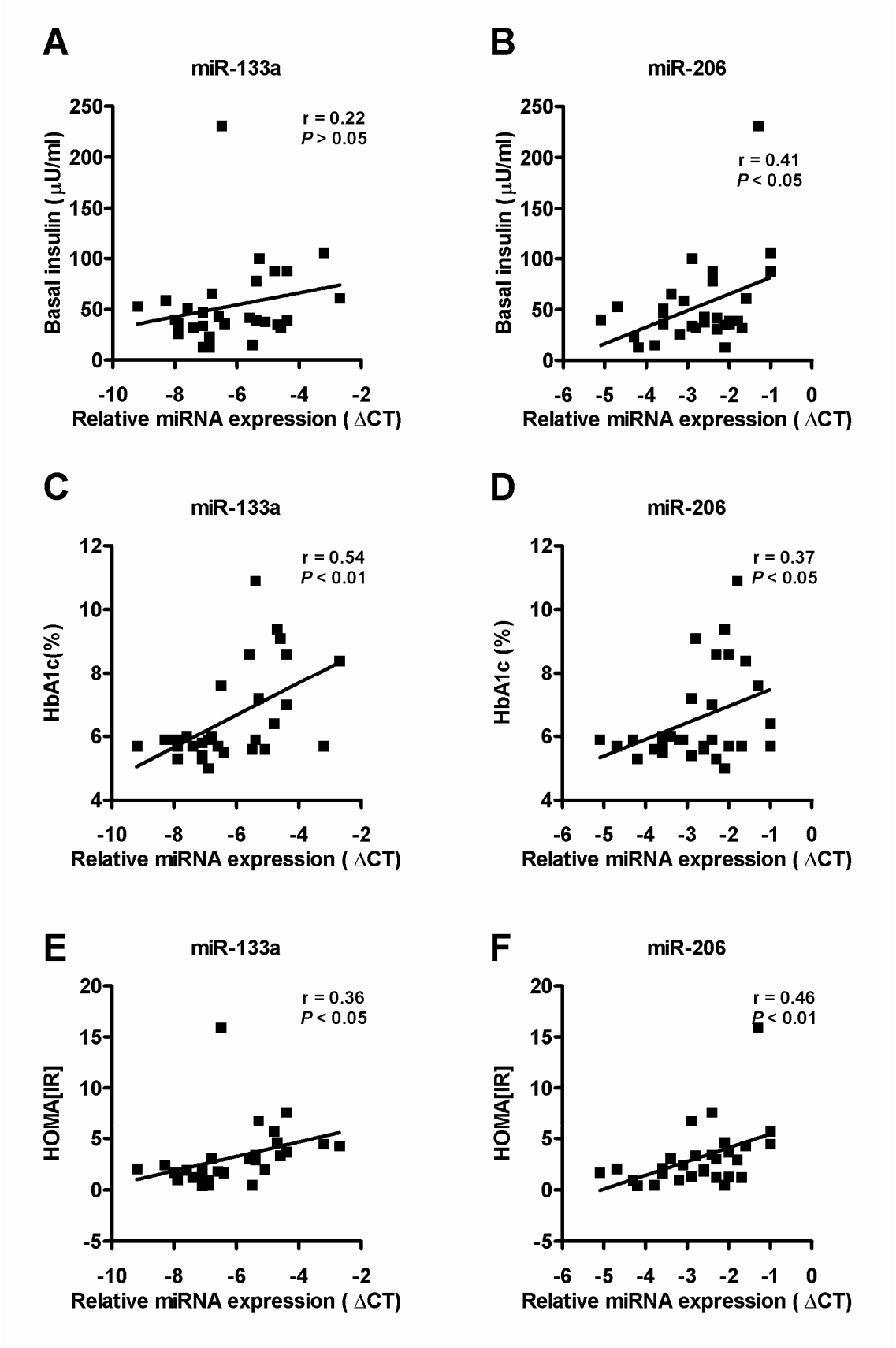
Supplementary Figure 4-4. Quantification of miR-133a expression from Northern confirmed down-regulation of miR-133a in Type 2 diabetes. Data shown as mean ± SE, n = 3 per group *$P < 0.05$



Supplementary Figure 4-5. Residual plots from multiple regression predicting (A and B) fasting glucose, (C and D) glucose tolerance, (E and F) HbA1c and (G and H) HOMA[IR].



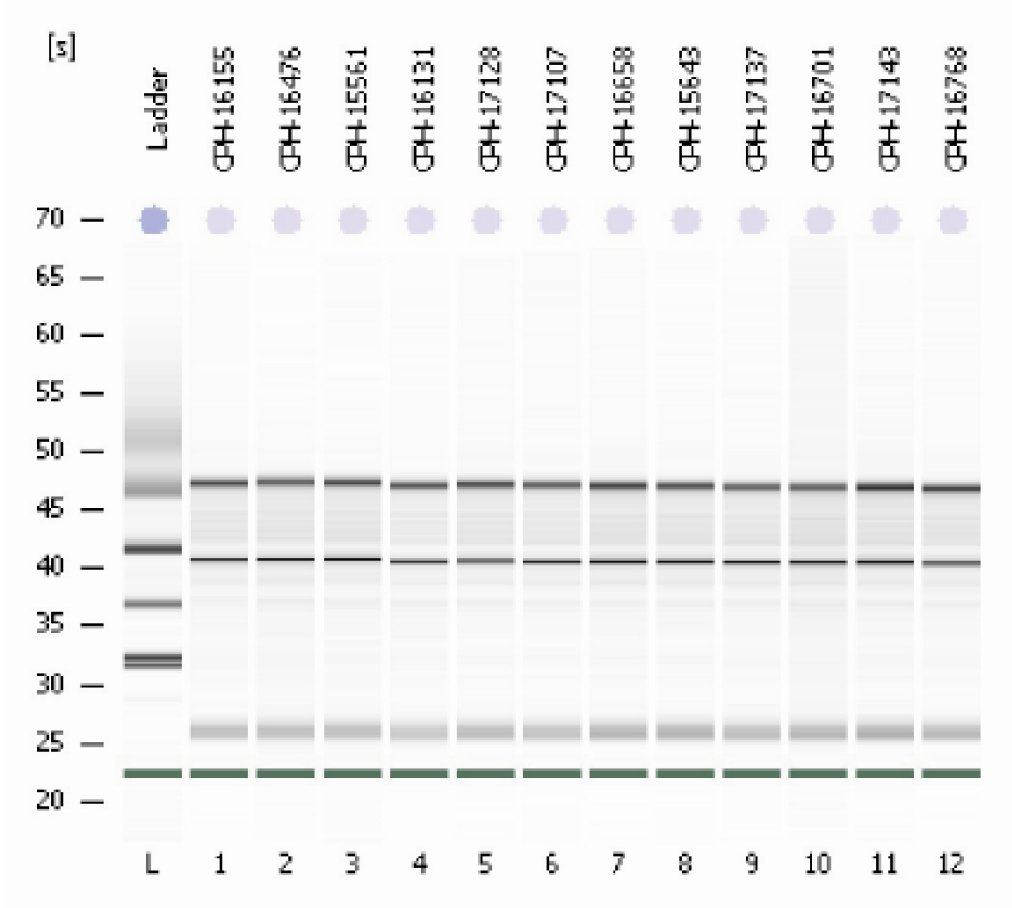
Supplementary Figure 4-6. Association between (A) miR-133a, (B) miR-206 and fasting glucose (mmol/L). Association between (C) miR-133a, (D) miR-206 and 2 h glucose tolerance (mmol/L).



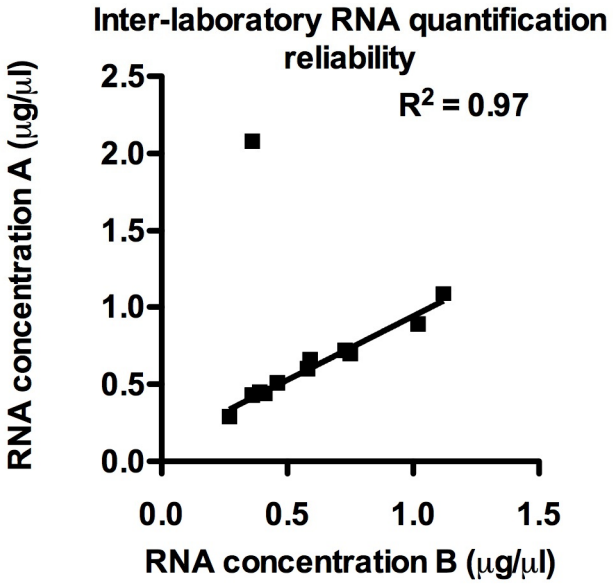
Supplementary Figure 4-7. Association between (A) miR-133a, (B) miR-206 and basal insulin. Association between (C) miR-133a, (D) miR-206 and HbA1c(%). Association between (E) miR-133a, (F) miR-206 and HOMA[IR].

Supplementary Table 4-2. miR-1/206 and miR-133 family sequence homology

microRNA	miRbase Accession	Sequence Homology
Hsa-miR-206	MIMAT0000462	5′-UGGAAUGUAAGGAAGUGUGUGG-′3
Hsa-miR-1	MIMAT0000416	5′-UGGAAUGUAAAGAAGUAUGUAU-′3
Hsa-miR-133a	MIMAT0000427	5′-UUUGGUCCCCUUCAACCAGCUG-′3
Hsa-miR-133b	MIMAT0000770	5′-UUUGGUCCCCUUCAACCAGCUA-′3



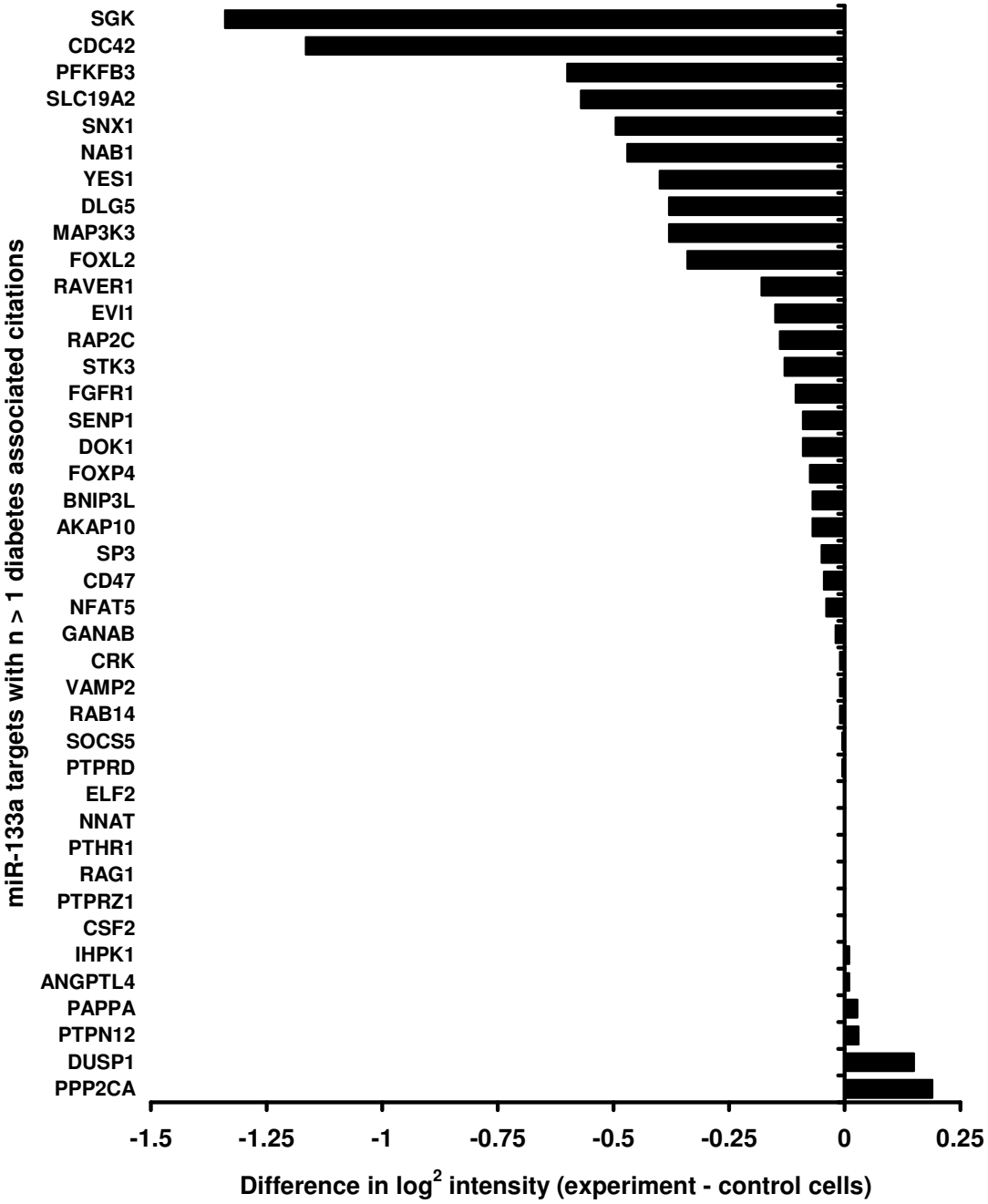
Supplementary Figure 4-8. Example of micro-electrophoresis gel showing RNA quality from Type 2 diabetes patient RNA samples. No evidence of RNA degradation and RIN scores were all >8.0



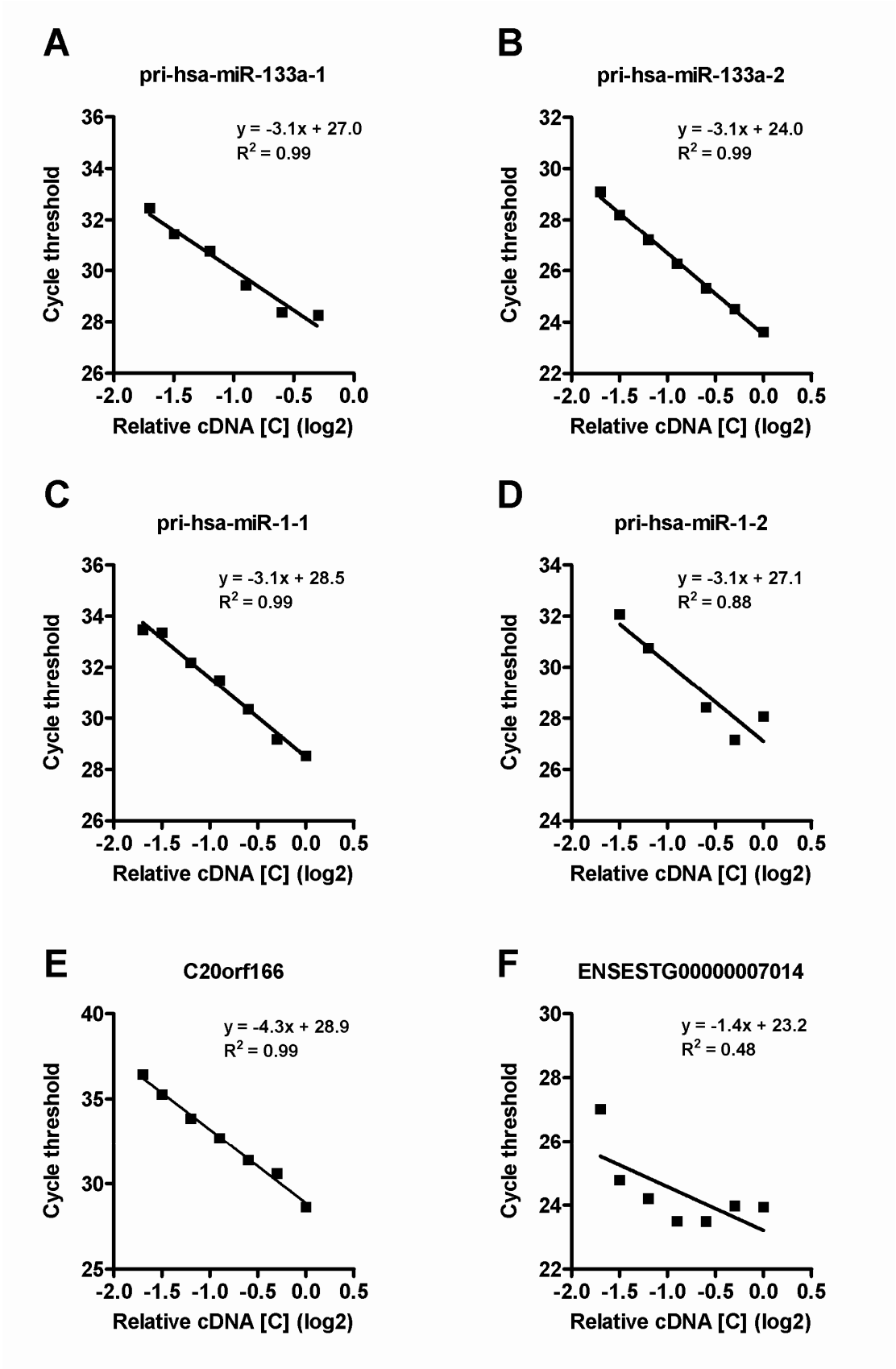
Supplementary Figure 4-9. Inter-laboratory RNA quantification reliability. RNA was quantified using a Nanodrop spectrophometer. The outlier is not included in the calculation of R^2

Supplementary Table 4-3. Inter-laboratory reliability of RNA quantification using a Nanodrop spectrophotometer. 260/230 ratio of <1.5 indicates possible phenol contamination during RNA isolation.

Sample ID	RNA quantification A (µg/µl)	RNA quantification B (µg/µl)	A260	A280	Ratio 260/280	Ratio 260/230
16494	0.72	0.73	18.301	9.219	1.99	0.84
16604	0.51	0.46	11.507	6.021	1.91	0.8
17110	0.29	0.27	6.739	3.702	1.82	0.55
17185	0.60	0.58	14.579	7.398	1.97	0.81
16482	0.45	0.39	9.8	5.119	1.91	0.63
17134	0.44	0.41	10.243	5.449	1.88	0.63
16440	0.43	0.36	8.922	4.732	1.89	1.13
15525	0.70	0.75	18.711	9.555	1.96	1.3
16795	2.08	0.36	8.93	4.745	1.88	1.34
15622	0.66	0.59	14.732	7.581	1.94	1.04
16407	1.09	1.12	28.093	14.134	1.99	0.79
15691	0.89	1.02	25.479	12.745	2	0.89



Supplementary Figure 4-10. Overexpression of miR-133a affects expression of miR-133a targets associated with diabetes. Data based on GEO8501 and curated literature citations linked to diabetes. Data represent difference in log^2 intensity between experiment and control cells.



Supplementary Figure 4-11. Efficiency of pri-microRNA primers. (A) Pri-miR-133a-1, (B) Pri-miR-133a-2, (C) Pri-miR-1-1, (D) Pri-miR-1-2, (E) C20orf166 (host gene) and (F) ENSESTG00000007014 (host gene) across a range of cDNA dilutions.

Supplementary Table 4-4. SAM analysis of microRNA target expression signatures.

microRNA	d. value	Raw p	Q value	microRNA	d. value	Raw p	Q value
hsa-let-7a	2.36	0.02	0.02	hsa-miR-1826	2.16	0.04	0.03
hsa-let-7b	2.36	0.02	0.02	hsa-miR-186	2.21	0.03	0.03
hsa-let-7c	2.36	0.02	0.02	hsa-miR-188-5p	2.26	0.03	0.03
hsa-let-7d	2.36	0.02	0.02	hsa-miR-18a	3.41	0.00	0.01
hsa-let-7e	2.36	0.02	0.02	hsa-miR-18b	3.41	0.00	0.01
hsa-let-7f	2.36	0.02	0.02	hsa-miR-190	2.25	0.03	0.03
hsa-let-7g	2.36	0.02	0.02	hsa-miR-190b	2.25	0.03	0.03
hsa-let-7i	2.36	0.02	0.02	hsa-miR-195	2.92	0.00	0.01
hsa-miR-1	2.48	0.02	0.02	hsa-miR-199a-3p	2.23	0.03	0.03
hsa-miR-101	2.89	0.00	0.01	hsa-miR-199b-3p	2.23	0.03	0.03
hsa-miR-106a	2.19	0.03	0.03	hsa-miR-19a	3.33	0.00	0.01
hsa-miR-106b	2.19	0.03	0.03	hsa-miR-19b	3.33	0.00	0.01
hsa-miR-1178	4.3	0.00	0.00	hsa-miR-202	3.59	0.00	0.00
hsa-miR-1185	2.31	0.02	0.03	hsa-miR-204	2.36	0.02	0.02
hsa-miR-1201	2.76	0.01	0.01	hsa-miR-206	2.48	0.02	0.02
hsa-miR-1227	2.81	0.01	0.01	hsa-miR-20a	2.19	0.03	0.03
hsa-miR-1231	2.56	0.01	0.02	hsa-miR-20b	2.19	0.03	0.03
hsa-miR-1236	2.96	0.00	0.01	hsa-miR-21	2.1	0.04	0.03
hsa-miR-124	3.86	0.00	0.00	hsa-miR-211	2.36	0.02	0.02
hsa-miR-1244	2.35	0.02	0.02	hsa-miR-216b	2.51	0.01	0.02
hsa-miR-1253	2.12	0.04	0.03	hsa-miR-221	2.69	0.01	0.02
hsa-miR-1258	2.92	0.00	0.01	hsa-miR-222	2.69	0.01	0.02
hsa-miR-1259	2.81	0.01	0.01	hsa-miR-223	3.33	0.00	0.01
hsa-miR-125a-3p	2.94	0.00	0.01	hsa-miR-25	2.13	0.04	0.03
hsa-miR-1264	2.55	0.01	0.02	hsa-miR-28-3p	2.5	0.01	0.02
hsa-miR-1270	4.09	0.00	0.00	hsa-miR-297	3.22	0.00	0.01
hsa-miR-1271	2.82	0.01	0.01	hsa-miR-301a	2.56	0.01	0.02
hsa-miR-1274a	2.82	0.01	0.01	hsa-miR-301b	2.56	0.01	0.02
hsa-miR-1274b	3.49	0.00	0.01	hsa-miR-302a	2.15	0.04	0.03
hsa-miR-127-5p	2.89	0.00	0.01	hsa-miR-302b	2.15	0.04	0.03
hsa-miR-1279	2.51	0.01	0.02	hsa-miR-302c	2.15	0.04	0.03
hsa-miR-1283	3.05	0.00	0.01	hsa-miR-302d	2.15	0.04	0.03
hsa-miR-1287	2.89	0.00	0.01	hsa-miR-302e	2.15	0.04	0.03
hsa-miR-1301	2.4	0.02	0.02	hsa-miR-30a	2.9	0.00	0.01
hsa-miR-1306	2.76	0.01	0.01	hsa-miR-30b	2.9	0.00	0.01
hsa-miR-130a	2.56	0.01	0.02	hsa-miR-30c	2.9	0.00	0.01
hsa-miR-130b	2.56	0.01	0.02	hsa-miR-30d	2.9	0.00	0.01
hsa-miR-1321	2.19	0.03	0.03	hsa-miR-30e	2.9	0.00	0.01
hsa-miR-1324	3.49	0.00	0.01	hsa-miR-31	2.28	0.03	0.03
hsa-miR-140-3p	2.79	0.01	0.01	hsa-miR-32	2.13	0.04	0.03
hsa-miR-143	2.59	0.01	0.02	hsa-miR-330-3p	2.55	0.01	0.02
hsa-miR-144	2.66	0.01	0.02	hsa-miR-331-5p	2.18	0.03	0.03
hsa-miR-148a	2.85	0.01	0.01	hsa-miR-338-5p	2.13	0.04	0.03
hsa-miR-148b	2.85	0.01	0.01	hsa-miR-339-5p	2.62	0.01	0.02
hsa-miR-150	2.49	0.02	0.02	hsa-miR-345	2.92	0.00	0.01
hsa-miR-151-3p	2.51	0.01	0.02	hsa-miR-363	2.13	0.04	0.03
hsa-miR-152	2.85	0.01	0.01	hsa-miR-367	2.13	0.04	0.03
hsa-miR-15a	2.93	0.00	0.01	hsa-miR-369-3p	2.86	0.00	0.01
hsa-miR-15b	2.93	0.00	0.01	hsa-miR-371-3p	2.22	0.03	0.03
hsa-miR-16	2.92	0.00	0.01	hsa-miR-372	2.15	0.04	0.03

hsa-miR-17	2.19	0.03	0.03	hsa-miR-373	2.15	0.04	0.03
hsa-miR-182	2.81	0.01	0.01	hsa-miR-374a	2.68	0.01	0.02
hsa-miR-1825	2.37	0.02	0.02	hsa-miR-374b	2.68	0.01	0.02
hsa-miR-520f	2.36	0.02	0.02	hsa-miR-585	2.19	0.03	0.03
hsa-miR-520g	2.46	0.02	0.02	hsa-miR-587	2.49	0.02	0.02
hsa-miR-520h	2.46	0.02	0.02	hsa-miR-590-5p	2.1	0.04	0.03
hsa-miR-521	3.2	0.00	0.01	hsa-miR-597	3.35	0.00	0.01
hsa-miR-522	2.2	0.03	0.03	hsa-miR-601	2.18	0.03	0.03
hsa-miR-524-3p	3.05	0.00	0.01	hsa-miR-603	2.43	0.02	0.02
hsa-miR-524-5p	2.92	0.00	0.01	hsa-miR-604	2.32	0.02	0.03
hsa-miR-525-3p	3.05	0.00	0.01	hsa-miR-605	2.68	0.01	0.02
hsa-miR-526a	3.06	0.00	0.01	hsa-miR-606	2.18	0.03	0.03
hsa-miR-526b	3.49	0.00	0.01	hsa-miR-610	2.67	0.01	0.02
hsa-miR-527	2.64	0.01	0.02	hsa-miR-620	4.09	0.00	0.00
hsa-miR-545	2.65	0.01	0.02	hsa-miR-622	2.35	0.02	0.02
hsa-miR-548a-3p	2.95	0.00	0.01	hsa-miR-628-3p	2.84	0.01	0.01
hsa-miR-548a-5p	2.93	0.00	0.01	hsa-miR-631	2.86	0.00	0.01
hsa-miR-548b-5p	2.93	0.00	0.01	hsa-miR-632	3.39	0.00	0.01
hsa-miR-548c-3p	2.35	0.02	0.02	hsa-miR-633	2.75	0.01	0.01
hsa-miR-548c-5p	2.93	0.00	0.01	hsa-miR-643	3.34	0.00	0.01
hsa-miR-548d-3p	2.38	0.02	0.02	hsa-miR-645	2.7	0.01	0.01
hsa-miR-548d-5p	2.93	0.00	0.01	hsa-miR-648	2.77	0.01	0.01
hsa-miR-548e	2.95	0.00	0.01	hsa-miR-649	2.72	0.01	0.01
hsa-miR-548f	2.95	0.00	0.01	hsa-miR-654-3p	2.23	0.03	0.03
hsa-miR-548g	2.65	0.01	0.02	hsa-miR-655	2.61	0.01	0.02
hsa-miR-548h	2.93	0.00	0.01	hsa-miR-720	2.55	0.01	0.02
hsa-miR-548i	2.93	0.00	0.01	hsa-miR-758	2.15	0.04	0.03
hsa-miR-548j	2.93	0.00	0.01	hsa-miR-765	2.83	0.01	0.01
hsa-miR-548k	2.5	0.01	0.02	hsa-miR-768-5p	2.73	0.01	0.01
hsa-miR-548l	2.8	0.01	0.01	hsa-miR-770-5p	2.98	0.00	0.01
hsa-miR-548m	3.34	0.00	0.01	hsa-miR-875-3p	2.57	0.01	0.02
hsa-miR-548n	2.38	0.02	0.02	hsa-miR-875-5p	2.76	0.01	0.01
hsa-miR-549	3.77	0.00	0.00	hsa-miR-885-5p	2.64	0.01	0.02
hsa-miR-550	2.76	0.01	0.01	hsa-miR-888	2.11	0.04	0.03
hsa-miR-556-5p	2.85	0.01	0.01	hsa-miR-889	2.12	0.04	0.03
hsa-miR-559	2.93	0.00	0.01	hsa-miR-891b	2.54	0.01	0.02
hsa-miR-563	2.4	0.02	0.02	hsa-miR-892a	2.1	0.04	0.03
hsa-miR-567	2.83	0.01	0.01	hsa-miR-922	2.73	0.01	0.01
hsa-miR-568	3.24	0.00	0.01	hsa-miR-92a	2.13	0.04	0.03
hsa-miR-570	2.4	0.02	0.02	hsa-miR-92b	2.13	0.04	0.03
hsa-miR-574-5p	3.17	0.00	0.01	hsa-miR-93	2.19	0.03	0.03
hsa-miR-575	2.18	0.03	0.03	hsa-miR-934	3.28	0.00	0.01
hsa-miR-577	2.31	0.02	0.03	hsa-miR-935	2.28	0.03	0.03
hsa-miR-580	2.66	0.01	0.02	hsa-miR-96	2.82	0.01	0.01
hsa-miR-581	2.61	0.01	0.02	hsa-miR-98	2.36	0.02	0.02

Supplementary Table 4-5. Fold change in microRNA probe expression between Type 2 diabetes and control groups from microRNA arrays based on pooled patient RNA (I. Gallagher, personal communication).

microRNA	Fold Change
hsa-miR-143/mmu-miR-143/rno-miR-143	1.31
hsa-miR-27b/mmu-miR-27b/rno-miR-27b	-1.30
hsa-miR-29a/mmu-miR-29a/rno-miR-29a	-1.20
hsa-miR-29b/mmu-miR-29b/rno-miR-29b	-1.10
hsa-miR-424	-1.90

Experimental validation was performed on these microRNAs using RT-qPCR and data is shown in 3.3.7.

Chapter 5 - MicroRNA knockdown and regulation in muscle cells

5.1. Introduction

MicroRNAs appear to change in skeletal muscle during the development of cancer cachexia and also Type 2 diabetes (Chapter 3 and 4), but the underlying mechanism and the functional consequences still need to be determined. The mechanism underlying the microRNA changes in Type 2 diabetes and cancer cachexia patients was not clear from the previous studies in Chapter 3 and 4. However, extracellular factors such as insulin or TNFα may play a role, as hormones and cytokines are known to activate or inhibit intracellular signaling pathways. For example, insulin and TNFα are known to modulate protein synthesis, protein breakdown and glucose uptake (Tisdale, 2005; Muoio & Newgard, 2008), which are important processes in Type 2 diabetes and cancer cachexia. From a functional viewpoint, there are many more predicted microRNA targets than experimentally validated microRNA targets (Bartel, 2009; Friedman et al. 2009; Lewis et al. 2003). Therefore it remains important to experimentally validate predicted targets of disease associated microRNAs, to establish a functional link between microRNAs and target proteins.

5.1.1 MicroRNAs are regulated by insulin and glucose

MicroRNAs are reported to respond to glucose and insulin in adipocytes and pancreatic β-cells (Poy et al. 2004; He et al. 2007; Ling et al. 2009; Tang et al. 2009). In insulin-resistant Type 2 diabetic rats with hyperglycaemia and hyperinsulineamia miR-29a was reported to be up-regulated in fat and skeletal muscle (He et al. 2007). In addition, high glucose and high insulin treatment reportedly up-regulates miR-29a in adipocytes (He et al. 2007). In Chapter 4, miR-29a was also found to be up-regulated in Type 2 diabetes patients, but miR-29a was not a strong predictor of fasting glucose, HbA1c, insulin resistance (HOMA[IR]) or glucose tolerance. However, miR-133a was found to be the strongest predictor of fasting glucose, HbA1c, insulin resistance (HOMA[IR]) and glucose tolerance of the microRNAs examined in Type 2 diabetes patients (Chapter 4). To date there have been no studies on whether skeletal muscle miR-133a expression changes in response to high glucose or insulin which are characteristics of Type 2 diabetes.

Recent findings from insulin-resistant adipocytes found miR-133a was among 29 down-regulated microRNAs (Ling et al. 2009) following high glucose (25 mmol/L) and high insulin (1 μmol/L) treatment. However, in another study in pancreatic β-cells, miR-133a was not among the sixty microRNAs detected to be modulated by extracellular glucose (Tang et al. 2009), which highlights microRNAs are not regulated in the same manner across all tissues. Therefore, the effects of extracellular factors on microRNA regulation in Type 2 diabetes should be examined primarily in muscle cells.

5.1.2 MicroRNAs may be regulated by TNFα

TNFα is known to trigger protein breakdown, thus has been implicated in the pathogenesis of cancer cachexia (Tisdale 2005; Stephens et al. 2008; Seruga et al. 2008). Micro-RNA-1, miR-133a and miR-206 were found to be down-regulated in pancreatic cancer cachexia patients (Chapter 3). Therefore it would be useful to study the effects of TNFα on miR-1, miR-133a and miR-206 expression in skeletal muscle. In addition, previously plasma TNFα has also been associated with Type 2 diabetes (Plomgaard et al. 2007). Treatment of myotubes with TNFα is reported to trigger insulin resistance and decrease glucose uptake (del Aguila et al. 1999). However, to date there have been no studies examining whether TNFα is a regulator of microRNA expression in skeletal muscle.

5.1.3 Approaches to determine the functional consequences of microRNAs

In Chapter 3 and Chapter 4 gene ontology enrichment analysis was used to identify functional gene groups, which may be coordinately targeted by microRNAs. For miR-133a, which was down-regulated in Type 2 diabetes patients, these functional gene groups included protein binding, intracellular organelles and protein amino acid dephosphorylation. To date no studies have been able to experimentally validate the targeting of functional gene groups, possibly due to the difficulty in measuring global protein changes.

Several studies have used both transcriptional and protein profiling in different tissues and identified potential microRNA targets based on discordant expression of gene and protein (Lu et al. 2008; Stentz & Kitabchi, 2007). For example, in pancreatic β-cells protein expression may increase while gene expression remains unchanged, providing an indication that a post-transcriptional mechanism may be determining protein levels (Lu et al. 2008). In skeletal muscle biopsies from Type 2 diabetes patients, mRNA and

protein levels have been measured simultaneously using 2D gels and microarrays (Stentz & Kitabchi, 2007). Discordant changes in mRNA and protein levels were reported (Stentz & Kitabchi, 2007), but only 80 proteins were quantified in contrast to ~40000 transcripts detectable by microarray and microRNAs were not measured. Nevertheless, changes in protein abundance in the absence of transcript changes suggest post-transcriptional regulation may be involved in Type 2 diabetes. However, without the sensitivity to quantify all proteins in skeletal muscle, it is difficult to confirm the targeting of functional gene groups by microRNAs. Thus functional microRNA studies tend to focus on one or two targets, using an *in-vivo* or *in-vitro* model to manipulate microRNA expression (Bartel, 2009).

5.1.4 In-vivo microRNA knockdown and overexpression

There is much interest in developing interventions to alter microRNA expression *in-vivo* (Mattes et al. 2008; Krützfeldt et al. 2005; Ren et al. 2009), but currently *in-vivo* manipulation of microRNA levels in patients has not been reported. *In-vivo* mouse studies have shown it is possible to deliver antisense microRNA oligonucleotides (ASOs) to the liver with functional consequences. ASOs conjugated with cholesterol and injected into mice were reported to significantly reduce miR-122 in liver (Krützfeldt et al. 2005). Targets of miR-122 include critical components of the cholesterol pathway, and knockdown of miR-122 *in-vivo* improved liver cholesterol (Krützfeldt et al. 2005). However, it remains to be seen whether microRNAs can be successfully delivered to other tissues such as skeletal muscle.

In our laboratory there is no facility for *in-vivo* mouse studies. Thus all data in this Chapter are based on *in-vitro* experiments using muscle cell cultures. This has advantages as many studies have shown microRNA expression levels can be rapidly manipulated *in-vitro*, thus allowing functional microRNA targets to be determined at the protein level as tissue availability is not an issue for *in-vitro* experiments.

5.1.5 In-vitro microRNA knockdown and overexpression

In-vitro miR-1 and miR-133a overexpression in the HeLa cell-line with low endogenous miR-1 and miR-133a expression combined with microarray profiling revealed many targets can be down-regulated both directly and indirectly (Lewis et al. 2003; Sethupathy et al. 2006). Subsequently, studies have often taken two approaches to determine microRNA function, studying loss of function or target protein expression

when a microRNA is over expressed, or gain of function or target protein expression when a microRNA is blocked.

Overexpression of microRNAs within a cell culture system can be achieved in several ways. The most commonly reported method is transfection of cells with microRNA duplexes, which are designed to mimic endogenous microRNAs following cleavage by DICER and results in suppression of targets with functional microRNA binding sites (Lim et al. 2005). Other options include using interventions earlier in the microRNA biogenesis pathway (Kim et al. 2009). Some studies have reported transfecting cells with microRNA precursor mimics. These microRNA precursor mimics can be processed by DICER to produce functional mature microRNAs, which leads to a suppression of valid microRNA target proteins (Lee et al. 2005). Another possibility is using a viral delivery system, by designing a vector containing the mature microRNA sequence of interest and infecting muscle cells (He et al. 2007). The advantage of using a viral delivery system is the ability to over-express the microRNA of interest for a longer period of time. Therefore, it would be possible to generate stable endogenous microRNA over-expressing cells, which would allow the longer-term consequences of elevated microRNA levels on cell phenotype to be observed.

In Type 2 diabetes and cancer cachexia miR-133a and miR-206 expression was down-regulated (Chapter 3 and 4). Therefore a microRNA knockdown model would be most relevant to experimentally validate miR-133a and miR-206 targets. For experimental validation of microRNA targets in skeletal muscle several cell-lines are available. In previous studies attempting to validate skeletal muscle microRNA targets the murine C2C12 cell-line has been used (Boutz et al. 2007; Chen et al. 2006; Kim et al. 2006; Rosenberg et al. 2006; Brzeszczynska et al. under revision). It is well known C2C12 myoblasts proliferate in the presence of serum and glucose, whereas in the presence of low serum C2C12 myoblasts differentiate and fuse into myotubes after around four days.

In the present study two approaches were attempted to knockdown mature miR-133a and miR-206 in myoblasts. First, pri-microRNA ASOs were designed to target upstream of the pre-miR-133a and pre-miR-206 hairpins in an attempt to specifically knockdown pri-miR-133a and pri-miR-206 transcripts. Second, mature microRNA ASOs designed to bind with perfect complementarity to their target mature microRNAs were used in an attempt to directly knockdown target mature microRNAs. It is

currently unknown if targeting pri-microRNAs is effective for specific knockdown of mature microRNAs (Davis et al. 2006). The latter approach is the most common, but the indirect effects on other microRNAs are rarely reported.

5.1.6 MicroRNA target validation

The targets chosen for experimental validation included PTBP1, CDC42, SMEK2 and TGIF2, which are predicted miR-133a and miR-206 targets. PTBP1 is an alternative splicing factor, which regulates the inclusion of exons in muscle development genes and suppression of PTBP1 leads to increased inclusion of target exons during muscle differentiation (Boutz et al. 2007). In cancer cachexia PTBP1 is potentially a relevant target of miR-133a, as *in-vitro* PTBP1 inhibits skeletal muscle differentiation. Therefore, the down-regulation of miR-133a with muscle-loss reported in Chapter 3 could be partly causing dedifferentiation via de-repression of PTBP1 protein levels.

CDC42 is a signal transduction kinase, which has been previously linked to insulin resistance and glucose uptake (Nevins and Thurmond, 2005; Cheatham et al. 1996). A direct interaction between VAMP2 and CDC42 has been reported which regulates SNARE-dependent vesicle movement (Cheatham et al. 1996; Nevins and Thurmond, 2005), which suggests both are potentially important for muscle glucose uptake. In addition, CDC42 is required for the activation of JNK signaling which is a negative regulator of insulin signaling (Coso et al. 1995). JNK protein is reportedly increased in skeletal muscle from obese Type 2 diabetes patients (Bandyopadhyay et al. 2005). CDC42 is a miR-133a target, which has been experimentally validated in cardiac muscle (Carè et al. 2007), but CDC42 has yet to be demonstrated as a miR-133a target in skeletal muscle.

SMEK2 and TGIF2 were the other predicted miR-133a targets selected for experimental validation. SMEK2 is a suppressor of MEK in the MAPK pathway, which is associated with multiple cellular functions including insulin signaling and muscle growth. However there are no studies on the function of SMEK2 in skeletal muscle. TGIF2 is a transcriptional repressor, which is reported to suppress genes regulated by the TGF-β transcription factors (Melhuish et al. 2001). Neither, SMEK2 nor TGIF2 has been previously associated with either Type 2 diabetes or cancer cachexia pathogenesis, thus validation of SMEK2 or TGIF2 as miR-133a targets would reveal a novel mechanism may contribute to Type 2 diabetes or cancer cachexia process.

5.1.7 Determination of microRNA function in skeletal muscle

When dysregulated *in-vivo* muscle specific microRNA expression appears to be altered in human diseases associated with insulin resistance. In Type 2 diabetes and pancreatic cancer cachexia patients the expression of mature miR-133a and miR-206 were both significantly reduced. Expression of these two microRNAs was also correlated with different stages of insulin resistance and the impairment of glucose tolerance potentially leading to Type 2 diabetes in the presence of pancreatic β-cell dysfunction. While miR-133a was reduced in patients with impaired glucose tolerance, it was reduced further in Type 2 diabetes patients. To determine the functional role of microRNAs within human Type 2 diabetes and cancer cachexia requires a model where microRNA expression can be manipulated and functional consequences determined.

5.1.8 Aims

- Determine the response of miR-133a and miR-206 in muscle cells to extracellular signals including insulin and TNFα.
- Evaluate the effectiveness of primary microRNA knockdown and mature microRNA knockdown in muscle cells.
- Determine the effects of miR-133a and miR-206 knockdown in muscle cells on predicted targets including CDC42, PTBP1, SMEK2 and TGIF.

5.2. Methods

5.2.1 Approach

Mature miR-1, miR-133a and miR-206 expression and primary microRNA transcription was determined in myotubes following insulin or TNFα treatment. Then to knockdown mature miR-133a and miR-206 two approaches were employed. Firstly, primary miR-133a and miR-206 transcripts were targeted by anti-sense LNA oligonucleotides (pri-microRNA ASOs) to determine their effectiveness. If primary knockdown of miR-133a and miR-206 transcripts is effective then this should also specifically reduce mature miR-133a and miR-206. Secondly, knockdown of mature miR-133a and miR-206 was attempted using ASOs designed to bind to mature microRNA. Mature microRNA ASOs were used for experimental validation of miR-133a and miR-206 targets. CDC42, PTBP1, TGIF2 and SMEK2 protein level was determined using Western blot in response to miR-133a and miR-206 knockdown.

5.2.2 Extracellular insulin, glucose and TNFα treatment

C2C12 myoblasts were seeded at low density in DMEM supplemented with 10% FBS and 1% Streptomycin and then grown until 70-80% confluence. Myogenic differentiation was induced when cells reached 70-80% confluence by changing cell media to high (25 mmol/L) or low glucose (5 mmol/L) DMEM supplemented with 10% horse serum. Day 4 differentiated myotubes were used to determine the affects of insulin on miR-133a, miR-206 and miR-1 expression. Immediately prior to experiments, cells were washed in 1 x PBS. Then myotubes were treated with 100 nM or 0.1 nM insulin (Sigma, UK) for 0, 10, 30 and 60 min in the presence of high (25 mmol/L) or low glucose (5 mmol/L), the experiment was performed in triplicate. To recover RNA, cells were lysed in TRIZOL as previously described in 5.2.4 then stored at -80°C prior to RNA isolation and quantification.

Mature miR-133a, miR-206 and miR-1 were measured in the cells using RT-qPCR as described in section 3.2.5 and 3.2.6 Unfortunately, RNA degradation occurred in the low glucose treated myotube samples and therefore RT-qPCR results from the myotubes treated with low glucose are not presented. To assess the effect of insulin (100 nM or 0.1 nM) on primary microRNA transcription, RT-qPCR was used to

measure expression of pri-miR-1-1, pri-miR-1-2, pri-miR-133a-1, pri-miR-133a-2, pri-miR-133b and pri-miR-206.

To determine the effect of TNFα on mature miR-1, miR-133a and miR-206 in skeletal muscle cells, C2C12 myoblasts were differentiated for 4 days as described and incubated in 10 ng/ml TNFα (Invitrogen, UK) for 0, 1 and 24 h. To recover RNA, cells were lysed in TRIZOL and stored at -80°C prior to analysis. These experiments were performed in triplicate but to reduce costs the microRNA analysis was conducted on duplicate samples in some cases, this is indicated in the results.

5.2.3 RNA isolation and quantification from muscle cells

The procedure for RNA isolation was similar to section 3.2.3 with the following modifications. Cell samples were defrosted at room temperature, 100 µl chloroform was added, samples were vortexed for 15 s and incubated for 10 min at 25°C. Then samples were centrifuged at 13000g for 15 min at 4°C. The aqueous phase was extracted and 250 µl isopropanol added. Samples were incubated for 10 min at 25°C then centrifuged at 13000g for 10 min at 4°C. The suspension was discarded and 1.5 ml 70% EtOH added, followed by centrifugation at 7500g for 15 min at 4°C. The suspension was again discarded and the pellet air-dried for 2 min. The RNA pellet was dissolved in 15 µl DEPC water. RNA concentrations were determined using a Nanodrop spectrophotometer as previously described (see section 4.2.5). Samples were stored at -80°C prior to further analysis.

5.2.4 Pri-microRNA knockdown

C2C12 mouse skeletal myoblasts were maintained in DMEM supplemented with 10% Fetal Bovine Serum and 1% penicillin/Streptomycin. C2C12 myoblast cells were seeded at 1 x 10^5 in 6 well plates 24 h prior to transfections. Immediately prior to transfections cells were washed in 1 x PBS and incubated in antibiotic free DMEM. Lipofectamine 2000 (Invitrogen, UK) and Optimem were used for all transfections. Pri-microRNA ASOs were designed to target 500 bp upstream and downstream of miR-133a-1, miR-133a-2 and miR-206. The microRNA ASO sequences are shown in Table 5-1. Two individual pri-microRNA ASOs were designed to target each pri-microRNA. The locked nucleic acids (LNA) modifications were used to increase stability of the pri-microRNA ASOs in the cell.

Table 5-1. Pri-microRNA ASO knockdown probe sequences

microRNA	pri-microRNA ASO sequence
Pri-miR-133a-1 A	5'- CACAgacagataCACA – 3'
Pri-miR-133a-1 B	5'- CCTGcctctgccTCCC –3'
Pri-miR-133a-2 A	5'- AAGGggaaatcaGAGG – 3'
Pri-miR-133a-2 B	5'- GGAAggggaaaaGCAG –3'
Pri-miR-206 A	5'- GAAAatgtagccAAGG –3'
Pri-miR-206 B	5'- AGGGtgcaggttgcAGGG –3'

pri-microRNA ASO knockdown probes were obtained from Exiqon, Denmark

Pri-microRNA ASO transfection in myoblasts

For transfections, pri-microRNA ASOs were mixed in 250 µl Optimem and seperately 5 µl Lipofectamine 2000 was mixed with 245 µl Optimem, then incubated for 5 min at 25°C. Pri-microRNA ASOs were then combined with Lipofectamine 2000 complexes and incubated for 25 min at 25°C. Transfections were initially performed three times in duplicate (n = 6) using pri-microRNA ASOs at 100 nM. For each transfection 500 µl transfection solution containing the pri-microRNA ASO/Lipofectamine/Optimem complexes was added dropwise to each well. Cells were harvested 48 h later for RNA and protein. Cells required for RNA were harvested in 500 µl TRIZOL and stored at -80°C until RNA isolation.

Knockdown of pri-microRNAs was measured using RT-qPCR as previously described (see section 4.2.8 - 4.2.9). Primers were designed to target the sequence within 500 base pairs of the pre-microRNA hairpin, primer sequences are listed in Table 5-2. Primer efficiency was determined by running RT-qPCR on five serial cDNA dilutions and plotting a standard curve to check linearity and slope >3.0 as described in section 4.2.7. Primer efficiency and linearity are shown in Supplementary Figure 5-1.

Table 5-2. Primers designed to amplify mouse pri-microRNA transcripts

Target	Primers, sequences or probes
Pri-mmu-miR-133a1	5'-tgtatatgcaagacattcttcaactg-3' (forward)
	5'-gagggaaatatctctacaaacatcaa-3' (reverse)
Pri-mmu-miR-1-2	5'-cccaaatcttgaagtagcctttag-3' (forward)
	5'-cgctcctctagtaaacctgcat-3' (reverse)
Pri-mmu-miR-1-1	5'-cactggtgagttggatcctg-3' (forward)
	5'-ggttctgtcctgtaccacagc-3' (reverse)
Pri-mmu-miR-133a-2	5'-ttgtctcctcaacaggcaag-3' (forward)
	5'-gggtcagtcacagcttaggg-3' (reverse)
Pri-mmu-miR-206	5'-ccctgaactcctcccttgt-3' (forward)
	5'-gagagagagagcatgaaattgga-3' (reverse)
Pri-mmu-miR-133b	5'-agctgccagtgtccattcat-3' (forward)
	5'-atcacctgcgtcacaatctg-3' (reverse)
Pri-mmu-miR-21	5'-ctttcttgctagtgtcctctgattt-3' (forward)
	5'-tcacctagagtgggaatctcttactt-3' (reverse)

Primers were obtained from Invitrogen UK

Following the initial transfections using 100 nM pri-microRNA ASOs, some resulted in cell death. Therefore, these pri-microRNA ASOs were transfected later at different concentrations, 10 nM, 30 nM, 50 nM and 100 nM. When pri-microRNA knockdown was evident, the downstream affects on mature microRNA expression were measured using RT-qPCR as described in section 3.2.5 and 3.2.6.

Pri-microRNA ASO transfection in myotubes

Additional experiments were conducted to determine whether pri-microRNAs could be knocked down effectively in myotubes, as typically myotubes are difficult to transfect, thus other methods such as electroporation have been used to deliver microRNA ASOs to myotubes (Chen et al. 2006), but indirect effects may occur during electroporation. Myoblasts were grown until 70-80% confluent in 6-well plates and myogenic differentiation was induced by changing cell media to DMEM supplemented with 10% horse serum. Cells were transfected with pri-microRNA ASOs at 100 nM after 4 days differentiation. Pri-microRNA ASOs were diluted in 250 µl Optimum and mixed gently. Separately, 4 µl Lipofectamine 2000 was mixed in 245 µl Optimum. After 5

min incubation at 25°C, pri-microRNA ASOs were combined with Lipofectamie 2000 and incubated for a further 25 min at 25°C. Cells were washed in 2 x PBS and 1 ml antibiotic-free DMEM was added to each well. The pri-microRNA ASOs were transfected dropwise to each plate. Cells were incubated at 37°C, after 12 h, cells were washed with PBS and new differentiation media added. Cells were harvested 48 h later and stored at -80°C until RNA isolation.

The results showed the pri-microRNA ASOs targeting pri-miR-133a-2 and pri-miR-206 did not appear to be as effective at achieving mature miR-133a and miR-206 knockdown compared to the mature microRNA ASOs described in the following section. Therefore validation of predicted miR-133a and miR-206 targets was carried out following transfection of the mature microRNA ASOs only.

5.2.5 Mature microRNA knockdown

C2C12 myoblast cells were seeded at 50% confluence and transfected with mature microRNA ASO targeting miR-133a and/or miR-206 (Exiqon, Denmark) at 100 nM with Oligofectamine (Invitrogen, UK) following the manufacturer's protocol. The mature microRNA ASO sequences are shown in Table 5-3. Prior to transfection, myoblast cells were incubated in antibiotic free DMEM. Mature microRNA ASOs were mixed with Optimem and then incubated with Oligofectamine for 25 min at 25°C. Transfections were performed in duplicate and myoblasts were not differentiated. Four hours after transfections, DMEM with Fetal Calf Serum was added to cells and after 48 h the cells were harvested.

To recover protein, cells were scraped in 1 x PBS and spun down. To recover RNA, cells were lysed in TRIZOL as previously described in section 5.2.3 then stored at -80°C prior to analysis. MicroRNA knockdown was confirmed using RT-qPCR as described previously in section 3.2.5 and 3.2.6. In addition, the affects of microRNA knockdown on mature miR-1, miR-133a, miR-206, pri-miR-1-1, pri-miR-1-2, pri-miR-133a-1 and pri-miR-133a-2 were quantified using RT-qPCR.

Table 5-3. Mature microRNA ASO probe sequences and product numbers

microRNA	Mature microRNA ASO probe	Product No.
mmu-miR-133a	CAGCTGGTTGAAGGGGACCAAA	139460-00
mmu-miR-206	CCACACACTTCCTTACATTCCA	139100-00
Scrambled control	GTGTAACACGTCTATACGCCCA	199002-00

Probes were obtained from Exiqon, Denmark

5.2.6 Western blot for detection of miR-133a and miR-206 targets

Western blot was performed to detect evidence of reduced suppression of target proteins CDC42, PTBP1, TGIF2 and SMEK2 in myoblasts cells following microRNA knockdown with mature microRNA ASOs. Cells were lysed at 100°C in Laemmli buffer for 5 minutes. Cells were centrifuged to remove insoluble material. BCA reagent was used to determine protein concentration. Proteins were heat-denatured and size fractionated using SDS-polyacrylamide gel electrophoresis. Proteins were loaded on a 4-12% gradient bis-Tris NuPage gel (Invitrogen, UK) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was stained with Ponceau Red to check the protein transfer (Supplementary Figure 5-5). The membrane was blocked using 5% skimmed milk in TBS, 0.2% Tween, 0.05% Triton X100 (TBST) at 25°C. Then membranes were incubated overnight at 4°C with primary antibodies diluted 1:1000 mixed with 5% BSA /TBST. Membranes were washed and incubated for 1 h at 25°C with an anti-rabbit IgG HRP conjugated antibody diluted 1:5000 (Cell Signaling Technology). Then blots were incubated in ECL reagent (GE Healthcare) and exposed to Kodak BioLight film. The protein signal on the blot was analysed using ImageJ software (NIH). The protein signal was corrected for protein loading based on the area under the curve from the Ponceau Red staining (Supplementary Table 5-1; Supplementary Figure 5-5). Loading corrected protein signals were then scaled to cells transfected with scrambled ASO.

5.2.7 Statistical Analysis

Statistical analysis was conducted in Prism v5.0 (GraphPad). To determine whether insulin and TNFα treatment of myotubes significantly decreased mature miR-1, miR-133a and miR-206 expression t-tests were conducted. Similarly, t-tests were used to determine whether the effect of miR-133a and miR-206 knockdown on protein targets was significant. Significance was accepted at $P < 0.05$.

5.3. Results

The response of miR-1, miR-133a and miR-206 expression in myotubes to insulin and TNFα treatment is presented in sections 5.3.1-5.3.3. To experimentally validate miR-133a and miR-206 targets potentially post-transcriptionally regulated in Type 2 diabetes and/or cancer cachexia at the protein level two approaches were used. In the first approach, pri-microRNA ASOs were designed in an attempt to knockdown mature miR-133a and mature miR-206, six pri-microRNA ASOs were tested for effectiveness and the results are presented in section 5.3.4. In the second approach two ASOs designed to target mature miR-133a and miR-206 were tested for effectiveness, the results are presented in section 5.3.7. The second approach proved most effective and was used to validate selected protein targets using Western blot in section 5.3.8.

5.3.1 Insulin affects mature miR-1 and miR-133a expression

Initially, the response of mature miR-133a was measured in myotubes in response to insulin resistance inducing high glucose and high insulin (100 nM) compared to control cells differentiated in low glucose media and treated with low insulin (0.1 nM), but RNA degradation occurred in the low glucose treated myotube samples (data not shown). Therefore, the results presented examine the response of microRNAs to high insulin (100 nM) only compared to untreated myotubes.

In response to 100 nM insulin miR-1 expression increased 40% after 30 min compared to untreated cells ($P < 0.05$) and this increase was also sustained at 60 min (Figure 5-1). There were no clear changes in miR-206 abundance in response to 100 nM insulin compared to untreated cells (Figure 5-1). There was a trend for insulin to decrease miR-133a expression around 20% after 60 min compared to untreated cells, but the decrease was not found to be significant (Figure 5-1).

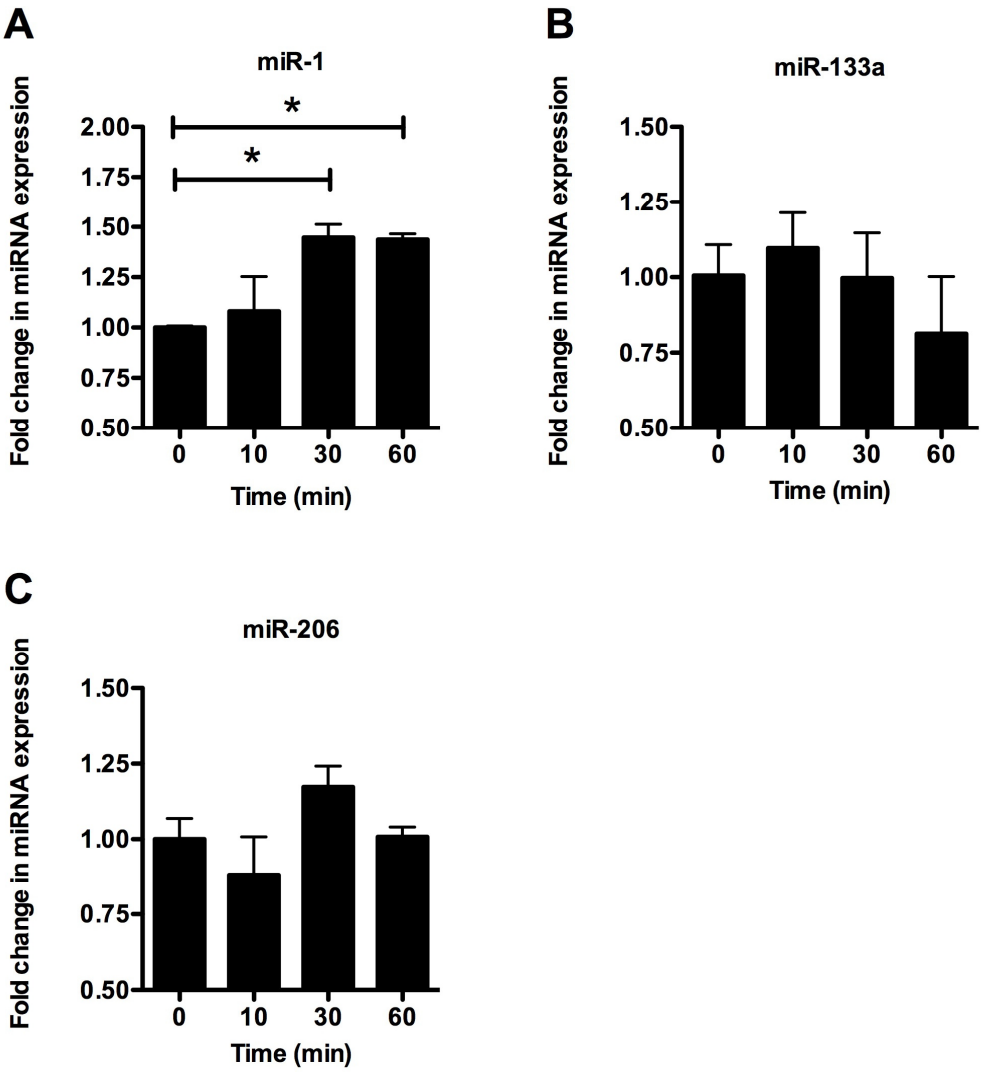


Figure 5-1. (A) Time-course of miR-1 (B) miR-133a and (C) miR-206 expression in response to 100 nM insulin treatment in myotubes (n = 3). Data shown as mean ± SE. * $P < 0.05$

5.3.2 Insulin stimulates primary microRNA transcription

The observed changes in mature microRNA abundance in C2C12 myotubes following insulin treatment led to the hypothesis that insulin may stimulate an increase in transcription of pri-microRNAs. MicroRNA-1 and miR-133a are transcribed from two genomic loci in human and mouse (Figure 5-2). Pri-miR-1-1 and pri-miR-1-2 are both processed to form mature miR-1, while pri-miR-133a-1 and pri-miR-133a-2 are processed to form mature miR-133a.

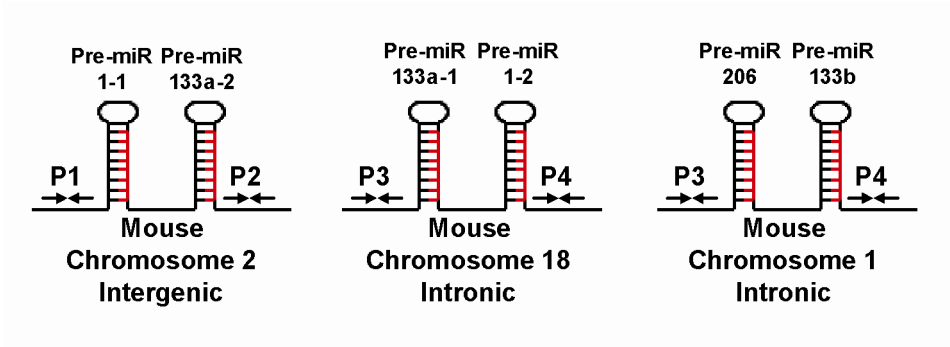


Figure 5-2. Genomic loci encoding miR-1/206 and miR-133 family. Arrows indicate location of primers designed to amplify the pri-microRNA transcript.

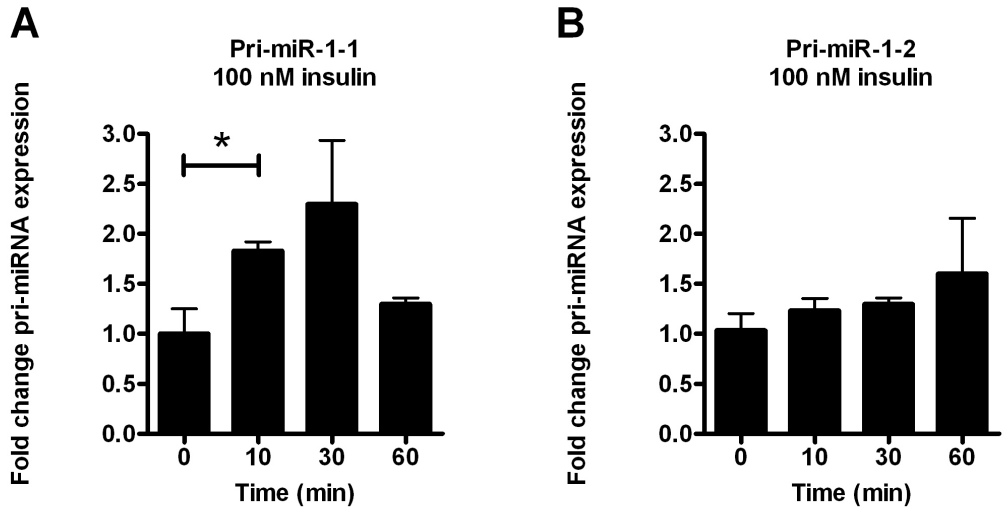


Figure 5-3. (A) Fold change in pri-miR-1-1 and (B) pri-miR-1-2 expression in response to 100 nM insulin treatment in myotubes. Data shown as mean ± SE. *$P < 0.05$

When myotubes were treated with 100 nM insulin, transcription of pri-miR-1-1 was significantly increased after 10 min ($P < 0.05$), and appeared to continue to increase up to 125% of basal after 30 min, while no significant changes were detected in pri-miR-1-2 transcription (Figure 5-3). To determine whether insulin concentration may influence pri-microRNA expression, myotubes were treated with a lower concentration of insulin (0.1 nM), transcription of pri-miR-1-1 and pri-miR-1-2 appeared to increase around 100% and 50% respectively after 60 min (Supplementary Figure 5-2).

In the previous section mature miR-133a expression appeared to decrease in response to insulin treatment, so if this change was linked to transcription one would hypothesize pri-miR-133a transcripts would be decreased as well. However, pri-miR-133a-1

transcript abundance was increased over 50% in response to 100 nM insulin after 10 min ($P < 0.05$) compared to untreated myotubes (Figure 5-4). There were no clear changes in pri-miR-133a-2 transcript abundance in response to 100 nM insulin. The changes observed in expression of pri-miR-133a transcripts did not appear to be influenced at lower insulin concentrations (Supplementary Figure 5-2).

Following the observation that mature miR-206 remained largely unchanged in myotubes in response to insulin treatment it would be expected that transcription of pri-miR-206 would also remain unchanged. On the contrary, pri-miR-206 expression followed a similar time course to pri-miR-1-2 in response to insulin (Figure 5-5). Pri-miR-206 transcription was increased over 100% after 60 min ($P < 0.05$) in response to 100 nM insulin treatment (Figure 5-5) and to a similar extent in response to 0.1 nM insulin treatment (Supplementary Figure 5-2).

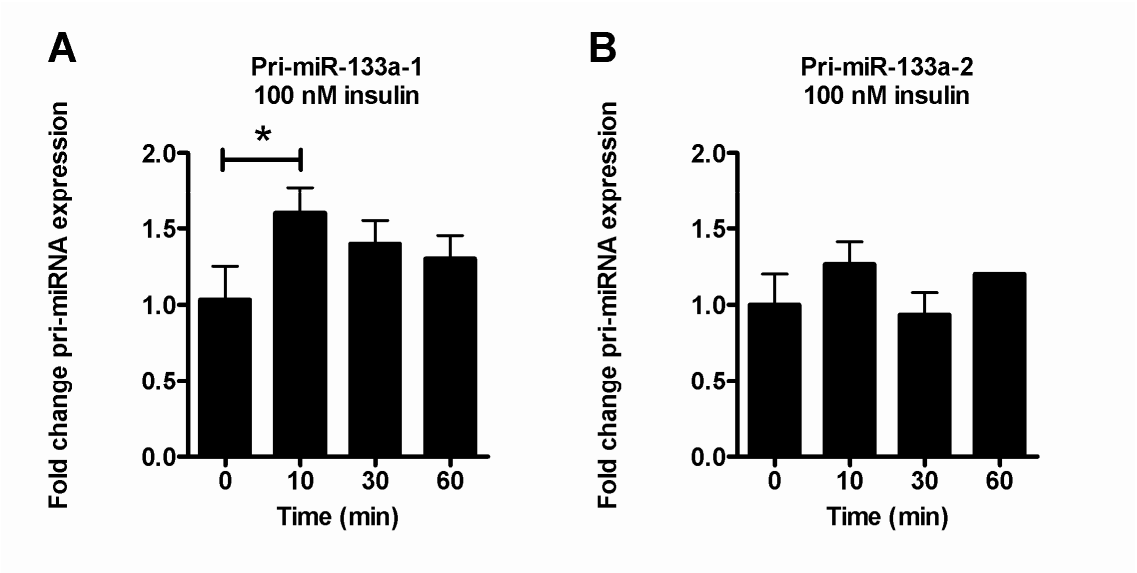


Figure 5-4. (A) Fold change in pri-miR-133a-1 and (B) pri-miR-133a-2 in response to 100 nM insulin treatment in myotubes. Data shown as mean ± SE. *$P < 0.05$

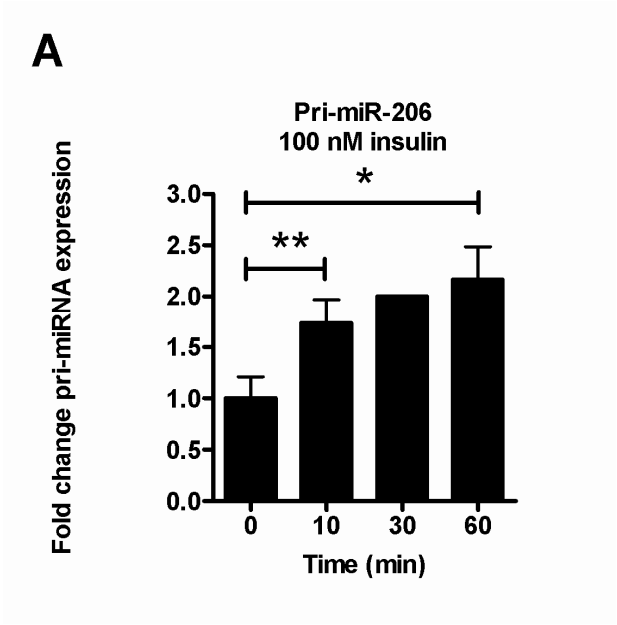


Figure 5-5. Fold change in (A) pri-miR-206 and (B) pri-miR-133b expression in response to 100 nM insulin treatment in myotubes. Data shown as mean ± SE. **$P < 0.01$ *$P < 0.05$

5.3.3 TNFα down-regulates mature miR-1, miR-133a and miR-206 expression

Cachexia and Type 2 diabetes are associated with elevated circulating TNFα levels. Therefore, differentiated myotubes were incubated in TNFα (10 ng/ml) for 1 h or 24 h. Myotubes were still visible following incubation with TNFα. It appeared miR-1, miR-206 and miR-133a levels responded to TNFα treatment (Figure 5-6). After 1 h a 20% decrease in miR-1 was observed, after 24 h miR-1 was reduced by 60% ($P < 0.01$) compared to control myotubes (Figure 5-6). A 50% reduction in miR-206 was clear after 1 h of TNFα treatment, following 24 h TNFα treatment miR-206 abundance was reduced by 70% ($P < 0.05$) compared to control myotubes (Figure 5-6). Finally, miR-133a was reduced by 40% after 1 h and 70% following 24 h TNFα treatment compared to untreated myotubes (Figure 5-6).

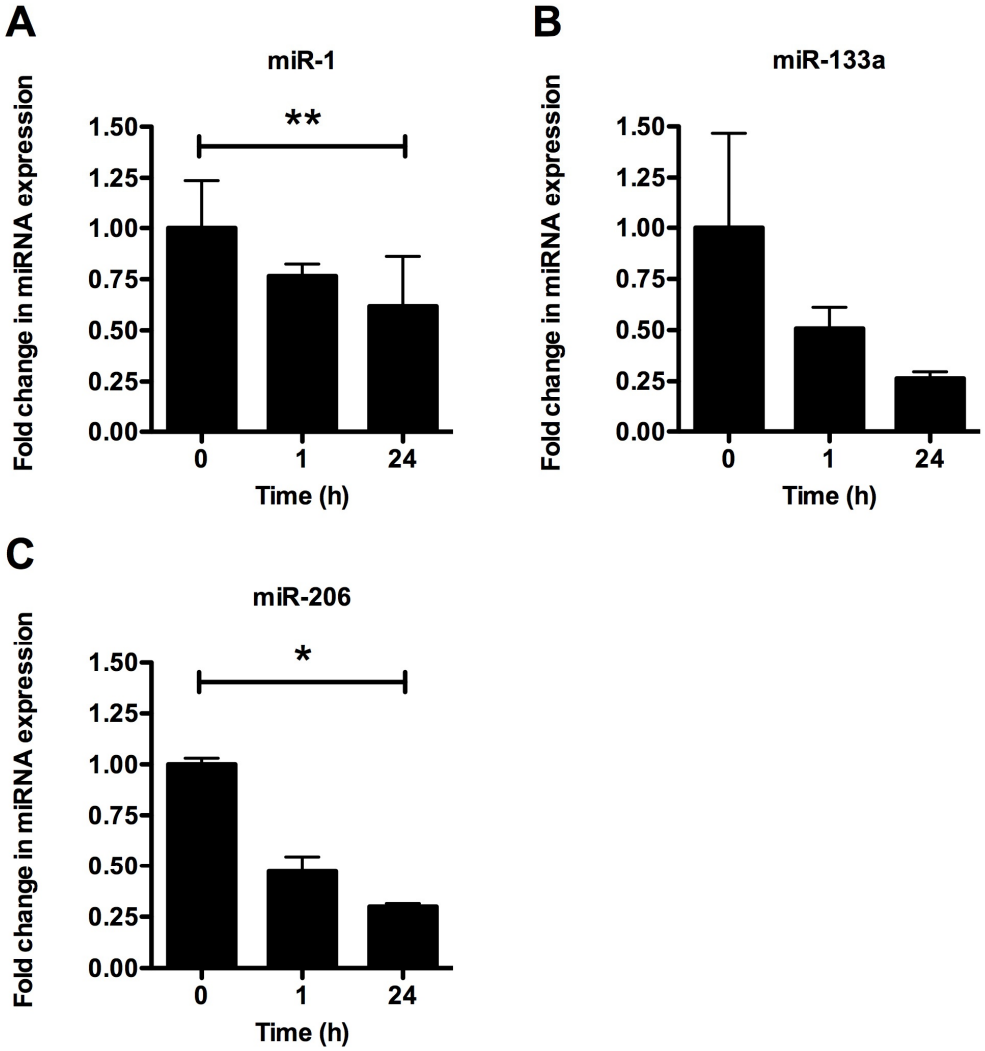


Figure 5-6. (A) Fold change in miR-1 (B) miR-133a and (C) miR-206 expression in response to TNFα treatment in myotubes. Data shown as mean ± SE. *****P* **< 0.01 ****P* **< 0.05

5.3.4 Effectiveness of pri-microRNA knockdown

Effective knockdown of pri-microRNA should lead to a decrease in mature-microRNA abundance. Two ASOs were designed to target upstream and downstream of the pri-miR-133a-1, pri-miR-133a-2 and pri-miR-206 respectively. Initially, the pri-microRNA ASOs were tested for effectiveness at 100 nM in myoblasts. Table 5-4 shows pri-miR-133a-1 ASO A and B proved to be ineffective at 100 nM as ΔCt and control ΔCt were similar and no significant changes were dectectable (see Supplementary Figure 5-3). Both pri-miR-133a-2 ASO A and B resulted in cell death (Table 5-4). Finally, pri-miR-206 ASO A resulted in a 1ΔCt change in pri-miR-206 expression, but pri-miR-206 ASO B resulted in cell death (Table 5-4).

Table 5-4. Initial screening of pri-microRNA ASO effectiveness in myoblasts

Pri-microRNA ASO	N	Concentration	ΔCt	Control ΔCt
Pri-miR-133a-1 ASO A	6	100 nM	-8.9	-8.7
Pri-miR-133a-1 ASO B	6	100 nM	-8.6	-8.7
Pri-miR-133a-2 ASO A	6	100 nM	Cells died	
Pri-miR-133a-2 ASO B	6	100 nM	Cells died	
Pri-miR-206 ASO A	6	100 nM	-6.7	-7.6
Pri-miR-206 ASO B	6	100 nM	Cells died	

Following the initial screening of pri-microRNA ASOs, three pri-microRNA ASOs were examined further at different concentrations. Pri-miR-133a-2 ASO A, pri-miR-133a-2 ASO B and pri-miR-206 B were transfected in myoblasts at 10 nM, 30 nM, 50 nM and 100 nM. Cell death was observed in myoblasts transfected with 100 nM pri-miR-133a-2 ASOs. Transfecting pri-miR-133a-2 ASO B at 10 nM, 30 nM and 50 nM reduced pri-miR-133a-2 expression by up to 80% ($P < 0.05$) although there appeared not to be directly corresponding effects on mature miR-133a expression (Figure 5-7). However, transfection of myoblasts with 30 nM or 50 nM pri-miR-133a-2 ASO B resulted in decreases in mature miR-133a of 40% ($P < 0.05$) and 80% ($P < 0.01$) respectively (Figure 5-7). Myoblasts transfected with 100 nM and 50 nM of pri-miR-206 ASO B resulted in cell death. Transfecting pri-miR-206 ASO B at lower concentrations reduced pri-miR-206 expression by 60-80% (Figure 5-8). Furthermore, mature miR-206 was reduced 70-75% when myoblasts were transfected with 10 nM or 30 nM respectively (Figure 5-8).

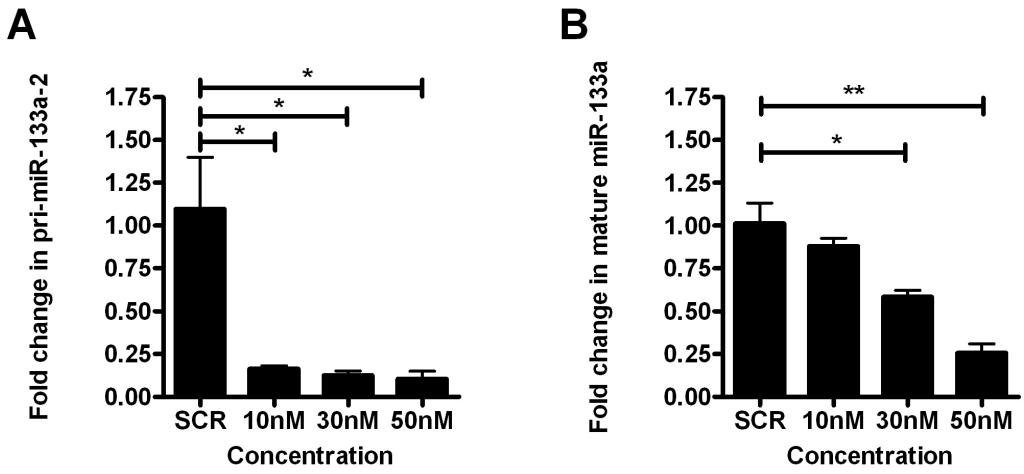


Figure 5-7. Pri-miR-133a-2 ASO transfection in C2C12 myoblasts at different concentrations. (A) Fold change in pri-miR-133a-2 in response to pri-miR-133a-2 ASO transfection at 10 nM, 30 nM, 50 nM compared to a scrambled ASO. (B) Fold change in mature miR-133a in response to pri-miR-133a-2 ASO transfection at 10 nM, 30 nM, 50 nM compared to a scrambled ASO. Data from triplicate transfections and shown as mean ± SE. **$P < 0.01$ *$P < 0.05$

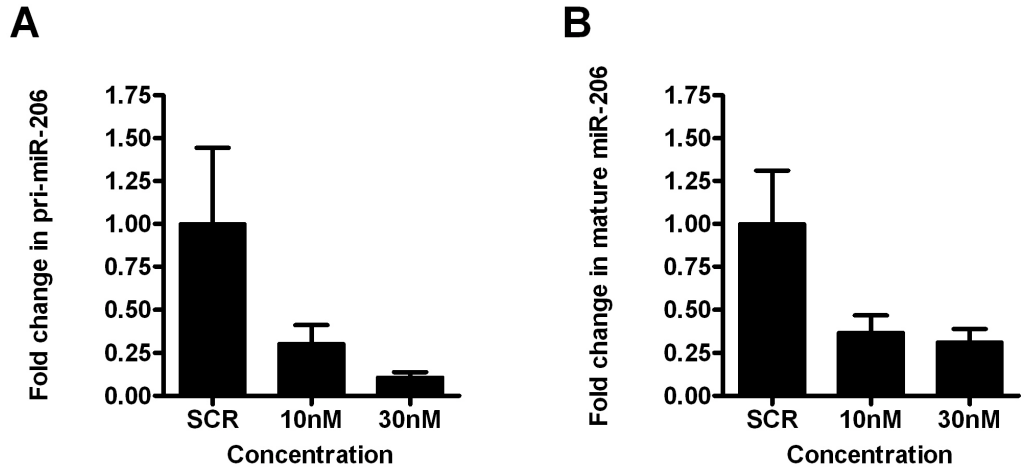


Figure 5-8. Pri-miR-206 ASO transfection in C2C12 myoblasts at different concentrations. (A) Fold change in pri-miR-206 in response to pri-miR-206 ASO transfection at 10 nM and 30 nM compared to a scrambled ASO. (B) Fold change in mature miR-206 in response to pri-miR-206 ASO transfection at 10 nM and 30 nM compared to a scrambled ASO. Data from triplicate transfections and shown as mean ± SE.

5.3.5 *Effectiveness of mature miR-133a and miR-206 knockdown*

Myoblasts transfected with mature microRNA ASOs targeting miR-133a resulted in knockdown of miR-133a to undetectable levels ($P < 0.05$, Figure 5-9), while there was no effect on miR-206 expression. In miR-206 ASO transfected myoblasts there was a 20% knockdown of miR-206 ($P < 0.05$, Figure 5-9). When miR-133a and miR-206 ASOs were transfected together, there was an 80% knockdown of miR-133a and miR-206 ($P < 0.05$, Figure 5-9). The housekeeping gene sno142 was stably expressed in myoblasts after transfection with miR-133a and miR-206 ASOs (Supplementary Figure 5-4). Mature miR-133a and miR-133b differ by a single nucleotide at their 3' end, so to check for specificity of miR-133a knockdown miR-133b expression was also measured. In miR-133a ASO transfected myoblasts there was also a 70% knockdown of miR-133b ($P < 0.05$, Figure 5-12). Both miR-133a and miR-133b share almost identical predicted mRNA targets so despite the lack of specificity of the miR-133a knockdown, target protein levels should still reflect functional consequences of miR-133 family knockdown.

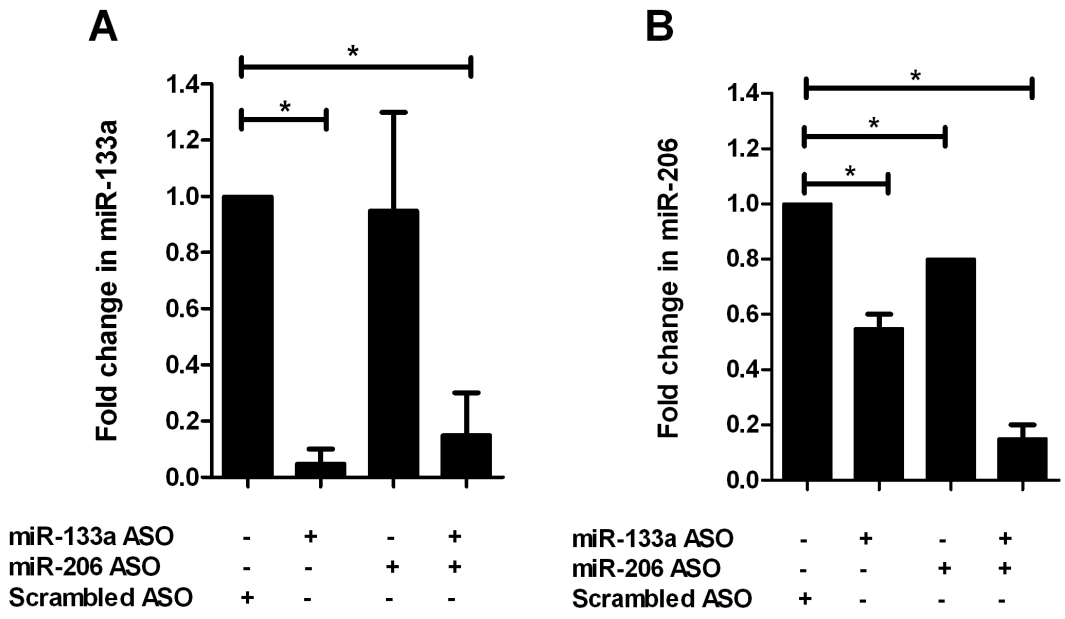


Figure 5-9. Fold change in mature (A) miR-133a and (B) miR-206 expression in response to mature miR-133a and miR-206 knockdown. Data based on duplicate transfections shown as mean ± SE. *$P < 0.05$

5.3.6 Knockdown of mature miR-133a and miR-206 regulates target proteins

Gene targets of miR-133a and miR-206 were selected for experimental validation by Western blot following knockdown using mature microRNA ASOs. The protein signal was normalized to total protein based on Ponceau Red staining of the nitrocellulose membrane rather than a housekeeping gene (Supplementary Table 5-1; Supplementary Figure 5-5). One-tailed t-tests were performed on duplicate cell samples to determine whether microRNA knockdown resulted in significant increases in target protein.

PTBP1 protein was increased around 20% following miR-133a knockdown ($P < 0.05$), while miR-206 knockdown resulted in a 20% decrease protein levels (Figure 5-10). There are four known PTBP1 isoforms, which the Western blot detects between 60-65 kDa. However, all share identical 3'UTRs and thus the same miR-133a and miR-206 binding sites. CDC42 was increased 125% following miR-133a knockdown ($P < 0.05$), while miR-206 resulted in 90% increase ($P < 0.05$) when compared to protein level in myoblasts transfected with a scrambled ASO (Figure 5-10).

SMEK2 was decreased around 50% following miR-133a knockdown ($P < 0.01$), miR-206 knockdown resulted in a 45% decrease ($P < 0.01$) in protein level (Figure 5-11). There was no additive effect evident following both miR-133a and miR-206 knockdown. Finally, TGIF2 results were less clear, miR-133a and miR-206 knockdown resulted in non-significant decreases around 40% compared to TGIF2 protein level in myotubes transfected with a scrambled ASO (Figure 5-11).

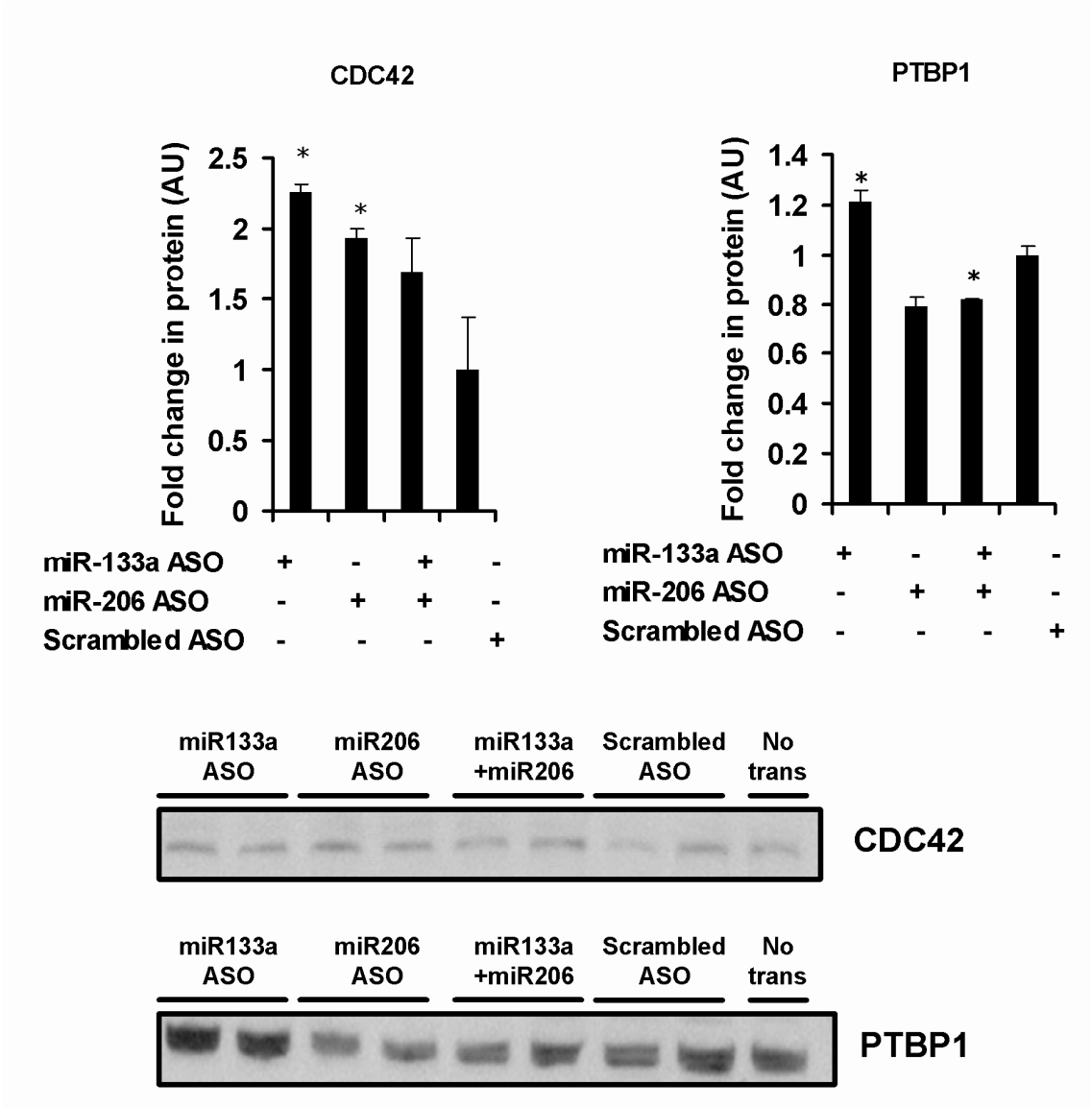


Figure 5-10. PTBP1 and CDC42 protein in response to miR-133a and miR-206 knockdown. Data from Western blot on duplicate transfected myoblasts. *P < 0.05 compared to scrambled ASO transfected myoblasts.

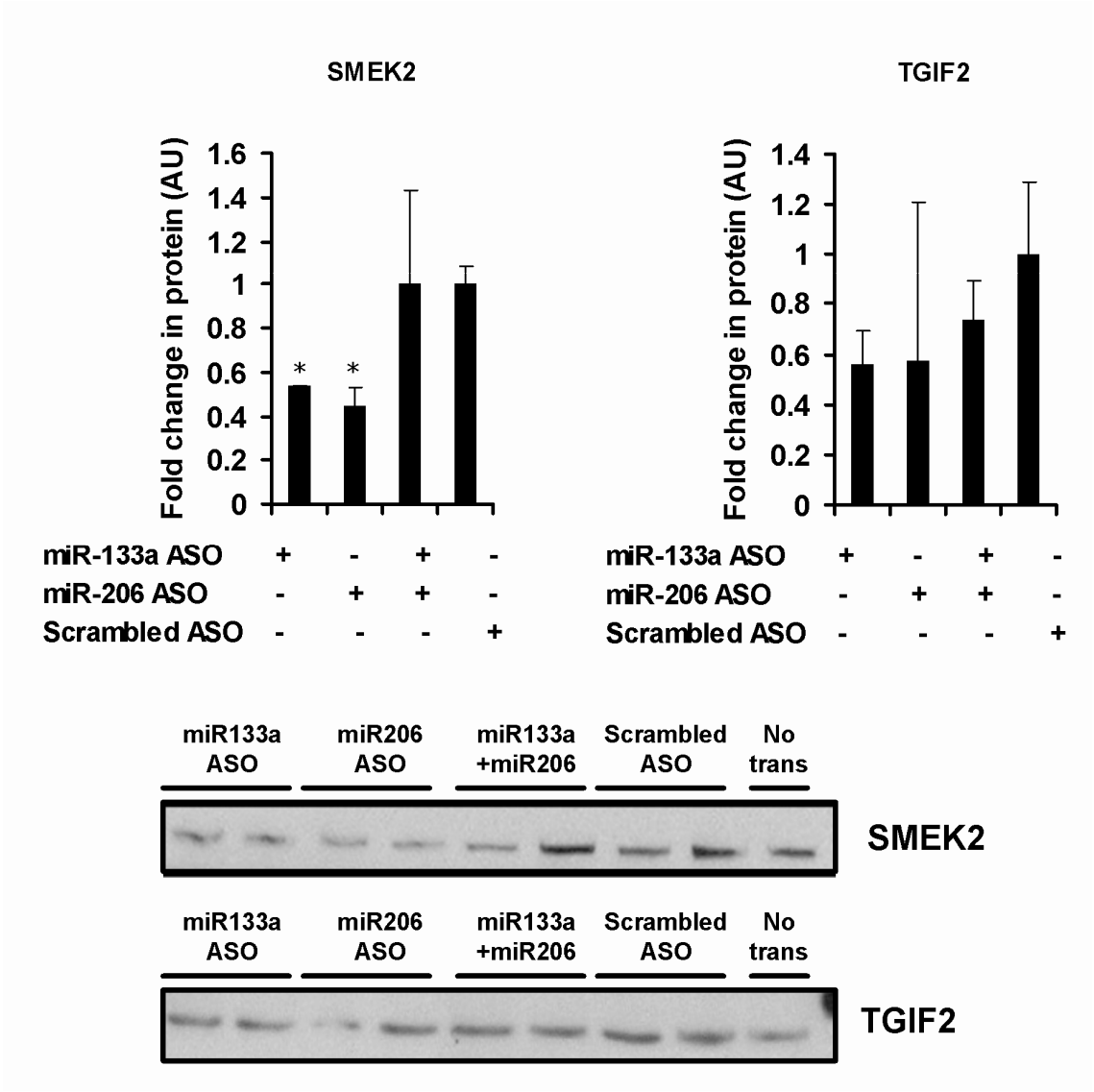


Figure 5-11. SMEK2 and TGIF2 protein in response to miR-133a and miR-206 knockdown. Data from Western blot on duplicate transfected myoblasts. *$P < 0.05$ compared to scrambled ASO transfected myoblasts.

5.3.7 Indirect affects of microRNA ASOs

Rarely are the effects of microRNA knockdown on other microRNAs reported. As miR-133a and miR-206 are both normally highly expressed in muscle and are predicted to target genes associated with transcriptional activation/suppression, it is possible that other microRNAs may be affected. Figure 5-12 shows miR-1 expression is suppressed around 30% following knockdown with miR-133a ($P < 0.05$) and miR-206. Remarkably, when both miR-133a and miR-206 were transfected together there was an 80% decrease in miR-1 expression ($P < 0.05$, Figure 5-12). Expression of miR-206 was affected by both miR-206 and miR-133a knockdown, again this effect appeared to be additive. When both miR-133a and miR-206 were transfected together this resulted in around an 80% decrease in miR-206 expression ($P < 0.05$, Figure 5-9). As described in section 5.3.5 miR-133a knockdown was very effective as miR-133a levels were found to be undetectable. Knockdown of miR-206 had no effect on miR-133a levels (Figure 5-9). Finally, miR-133b levels were reduced by around 70% following miR-133a knockdown, indicating the mature microRNA ASOs appeared to have unspecific affects on other muscle specific microRNAs ($P < 0.05$, Figure 5-12).

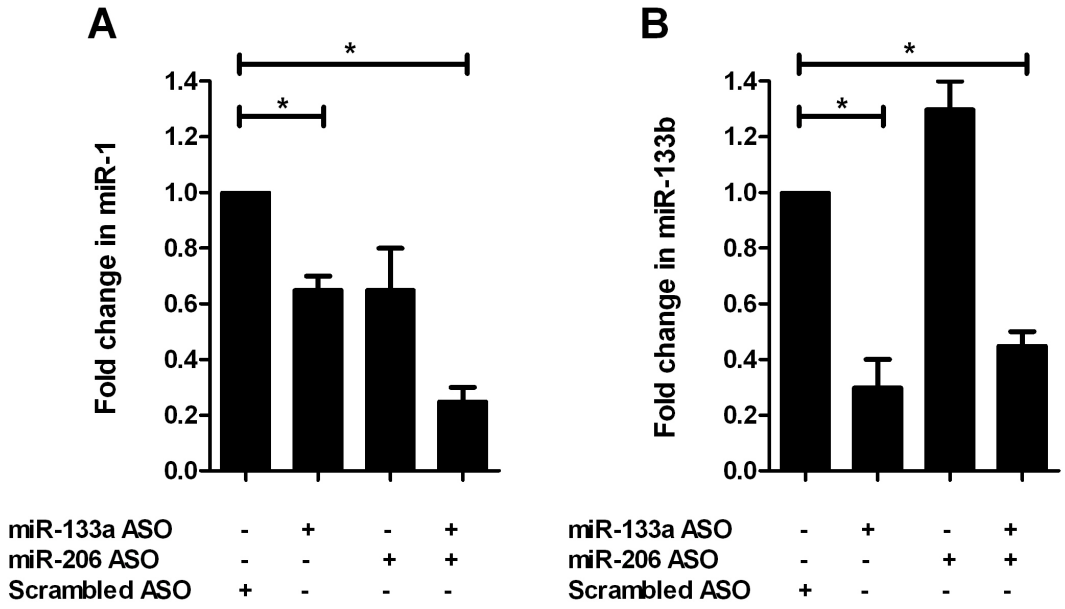


Figure 5-12. Fold change in mature (A) miR-1 and (B) miR-133b expression in response to mature miR-133a and miR-206 knockdown. Data shown as mean ± SE based on duplicate transfections. *$P < 0.05$

5.3.8 *Effects of microRNA ASOs on pri-microRNA transcription*

The observation that miR-1 and miR-206 expression appeared to be reduced following miR-133a or miR-206 knockdown raises the questions whether this change was occurring at the level of transcription. Using RT-qPCR the expression of pri-microRNA encoding miR-1, 206 and miR-133 families was examined in myoblasts following miR-133a or miR-206 ASO knockdown. Transcription of pri-miR-133a-1 and pri-miR-133a-2 was not significantly affected by miR-133a or miR-206 knockdown (Figure 5-13). Transcription of pri-miR-1-2 ($P < 0.05$) was increased after miR-206 knockdown, but pri-miR-1-1 and pri-miR-133b were not significantly affected (Supplementary Figure 5-6). The housekeeping gene 18S was stably expressed following mature ASO transfections (Supplementary Figure 5-4)

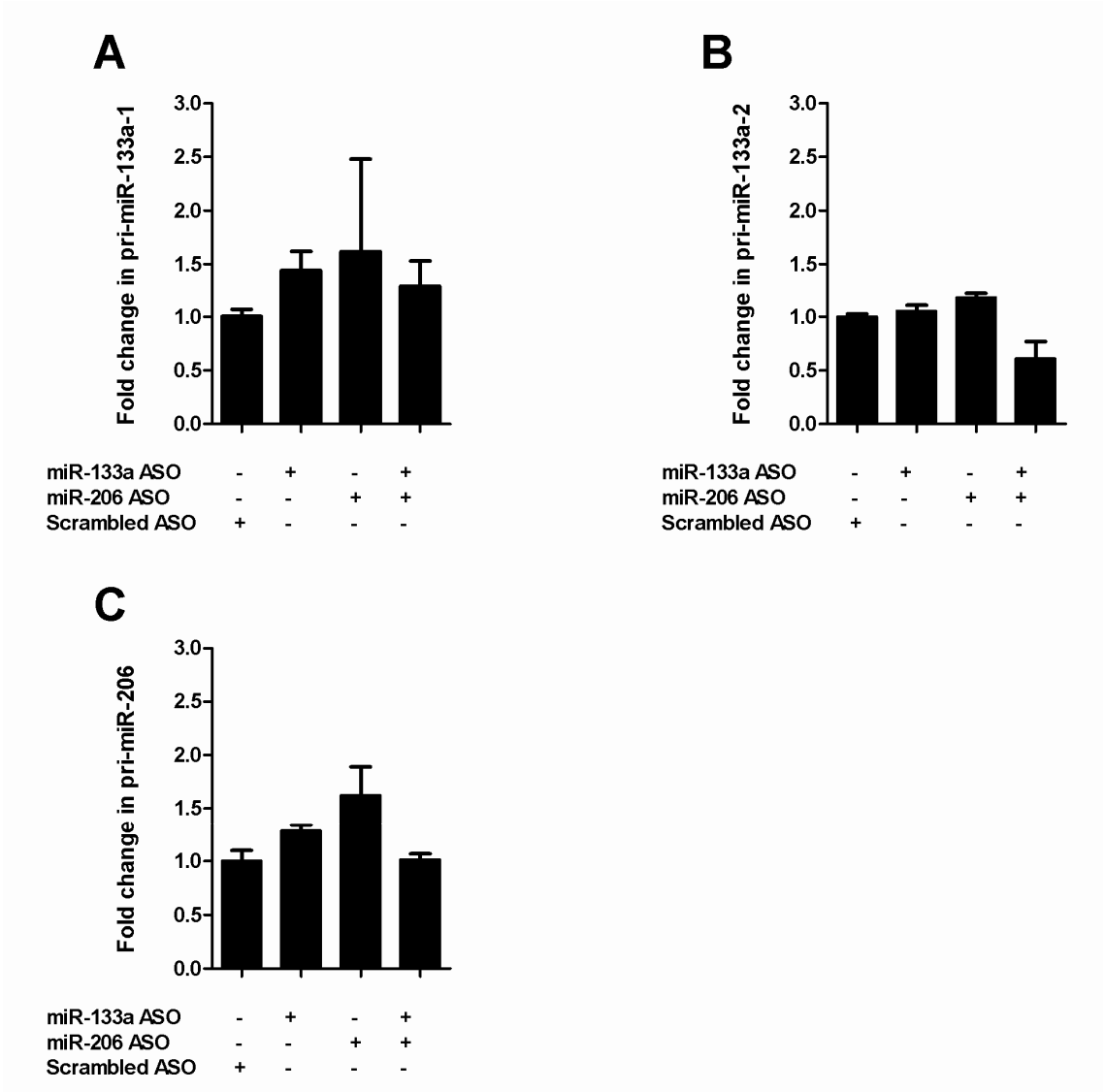


Figure 5-13. Effect of miR-133a and miR-206 knockdown on transcription of (A) pri-miR-133a-1, (B) pri-miR-133a-2 and (C) pri-miR-206. Data from duplicate transfections shown as mean ± SE.

5.4. Discussion

MicroRNA-1, miR-133a and miR-206 expression in myotubes responds to insulin and TNFα, which may partly explain the microRNA changes observed in Type 2 diabetes and pancreatic cancer cachexia patients (Chapter 3 and Chapter 4). However, insulin appeared to stimulate primary miR-1, miR-133a and miR-206 expression and these changes were not consistent with changes in mature microRNA levels. In order to experimentally validate targets microRNA knockdown was attempted using two approaches knockdown of primary miR-133a/206 and knockdown of mature miR-133a/206. CDC42 and PTBP1 were confirmed as miR-133a targets in skeletal muscle myoblasts, but TGIF2 and SMEK2 appeared not to be miR-133a or miR-206 targets. However, there were indirect effects of mature miR-133a and miR-206 knockdown using mature microRNA ASOs on primary and mature miR-1, miR-133a and miR-206 expression. More specific knockdown of mature miR-133a and miR-206 was achieved by targeting pri-miR-133a-2 and pri-miR-206, but requires further optimisation.

5.4.1 Insulin affects primary and mature microRNA expression in myotubes

Type 2 diabetes is characterised by hyperinsulinaemia and hyperglycaemia. In this study myotubes were incubated in 100 nM insulin in the presence of 25 nM glucose and the effects on miR-1, miR-133a and miR-206 expression measured at 0, 10, 30 and 60 min. Despite the relatively short time frame miR-1 increased, there was a non-significant decrease in miR-133a, while miR-206 remained stable, but the mechanism could not be determined in the present study. The results support the idea that the down-regulation of miR-133a observed in Type 2 diabetes patients may in part be due to the effects of hyperinsulinaemia. However, it would be worthwhile to repeat the cell experiments for different time points to determine whether chronic insulin treatment over 24-48 h down-regulates miR-133a.

No transcriptional changes in pri-miR-133a-1 or pri-miR-133a-2 were observed in the Type 2 diabetes patients (Chapter 4). Therefore it was important to establish whether hyperinsulinaemia had any effect on primary miR-133a transcription in the myotubes. Expression of pri-miR-133a-1 increased after 10 min in response to 100 nM insulin, but 100 nM insulin had no effect on pri-miR-133a-2. Expression of pri-miR-206 also increased in response to 100 nM insulin but had no effect on its neighbouring transcript pri-miR-133b. Expression of pri-miR-1-1 increased in response to 100 nM insulin, but expression of pri-miR-1-2 was unchanged. There appeared to be discordant expression

of primary and mature miR-133a and miR-206. While mature miR-133a was decreased, primary miR-133a-1 expression appeared to be increased. Likewise, pri-miR-206 expression was increased, but mature miR-206 was unchanged.

Past studies that have examined the effect of insulin on microRNA expression have been restricted to pancreatic β-cell-lines or adipocytes (Poy et al. 2004; He et al. 2007; Ling et al. 2009; Tang et al. 2009) and have not considered the effect of insulin on primary microRNA transcription. The present data suggest further regulation occurs in the microRNA biogenesis and processing pathway, which determines functional mature microRNA expression. However, it was not possible in the present study to examine the mechanism by which insulin influences primary and mature miR-1, miR-133a and miR-206 expression.

5.4.2 TNFα decreases muscle-specific microRNA expression in myotubes

In Type 2 diabetes patients TNFα has been reported to be elevated, and is a factor which may impair insulin signaling and hence muscle glucose uptake (Plomgaard et al. 2007; Ruge et al. 2009). In addition, TNFα is known to trigger protein breakdown in skeletal muscle, thus has been implicated in the pathogenesis of cancer cachexia (Tisdale 2005; Stephens et al. 2008; Seruga et al. 2008). In the present study, the effect of TNFα on miR-1, miR-133a and miR-206 expression was examined to determine whether TNFα may explain some of the changes in miR-1, miR-133a and miR-206 expression observed in human skeletal muscle in Chapter 3 and Chapter 4. Treatment of myotubes with TNFα led to a decrease in miR-1, miR-133a and miR-206 expression after 1 h. TNFα treatment for 24 h resulted in a 60-70% decrease in miR-1, miR-133a and miR-206 expression. These findings suggest TNFα may be a regulator of muscle specific microRNA expression, but the mechanism could not be determined in the present study. Further data would be useful from an *in-vivo* human TNFα infusion study to verify muscle specific microRNA expression responds to TNFα *in-vivo*. A practical approach would be to examine the effects of blocking TNFα on microRNA expression in sepsis patients with chronic inflammation.

5.4.3 Effectiveness of pri-microRNA and mature microRNA knockdown

Mature microRNA sequences are contained within structured hairpins in pri- and pre-microRNA (Lau et al. 2001). It has been reported ASOs tend to have reduced

effectiveness when targeting structured hairpins (Davis et al. 2006). Nevertheless, targeting of pre-microRNA using siRNA has been reported in cancer cell lines, but high concentrations were required for effective knockdown of mature miR-125b (Lee et al. 2005). Targeting pre-microRNA for knockdown of mature miR-133a was not an ideal approach in this study as Chapter 4 showed pre-miR-133a appeared to be rapidly processed in skeletal muscle from Type 2 diabetes patients, thus would be difficult to target effectively. Therefore the approach taken in the present study was to target pri-microRNA transcripts outside of the pre-microRNA hairpin structure (Davis et al. 2006). It must be borne in mind however, that the effectiveness of targeting pri-microRNA using ASOs has not been previously reported in skeletal muscle.

In this study, ASOs targeting pri-miR-133a-1, pri-miR-133a-2 and pri-miR-206 were transfected into myoblasts to determine their effectiveness to knockdown pri-miR-133a-1, pri-miR-133a-2 and pri-miR-206. Both ASOs designed to target pri-miR-133a-1 were ineffective at 100 nM for reducing pri-miR-133a-1 expression after 48 h. This may be because pri-miR-133a-1 has a long half life in skeletal muscle, although the half-life of pri-microRNAs has not been reported in muscle or indeed any other tissue. If miR-133a-1 does have a long half-life then 48 h may have been too early to detect knockdown. The pri-microRNA ASOs targeting pri-miR-133a-2 and pri-miR-206 appeared to be effective at reducing pri-microRNA expression and thus mature microRNA expression when transfected at lower concentrations.

In myoblasts pri-miR-133a-2 expression was effectively reduced by 80% by transfection of pri-miR-133a-2 ASOs at 10 nM, 30 nM and 50 nM. Interestingly, mature miR-133a was not significantly reduced by pri-miR-133a-2 ASO transfections at 10 nM despite the 80% knockdown of pri-miR-133a-2. Pri-miR-133a-2 ASO transfections at 30 nM resulted in 40% knockdown of mature miR-133a. While pri-miR-133a-2 ASO transfections at 50 nM resulted in a comparable 80% knockdown of mature miR-133a. Transfection of pri-miR-206 ASOs at 10 nM and 30 nM resulted in 60-80% knockdown in pri-miR-206 and 60-80% knockdown of mature miR-206 at 30 nM after 48 h. However, transfection of pri-miR-206 ASOs at 50 nM resulted in cell death.

In the present study the mechanism by which the pri-microRNA ASOs were acting was not determined, but it has been suggested that ASOs may act via an RNase H-based

mechanism causing degradation of the target transcript (Davis et al. 2006). Together these findings suggest targeting of pri-miR-133a-2 and pri-miR-206 can be effective for specifically reducing mature miR-133a and mature miR-206. This could be useful for further experiments to validate miR-133a and miR-206 targets. Preliminary experiments suggest it may be possible to knockdown pri-miR-133a-2 and pri-miR-206 in myotubes using ASOs (data not shown). In addition, the knockdown effects of pri-miR-206 in myoblasts appear to be retained upto 48 h during differentiation. Therefore, targeting of pri-miR-133a-2 and pri-miR-206 could be useful for determining the contribution of these transcripts to myogenic differentiation (Brzeszczynska et al. under revision). Furthermore, pri-microRNA ASOs could be useful for specific knockdown of mature microRNAs from the same family, but derived from different primary transcripts.

The most common approach reported to knockdown microRNAs in muscle is by transfection of ASOs directly targeting mature microRNAs. Therefore the effectiveness of mature microRNA ASOs targeting mature miR-133a and mature miR-206 was determined in myoblasts. Mature miR-133a ASO transfection at 100 nM resulted in undetectable levels of miR-133a in myoblasts after 48 h. Transfection of a miR-206 ASO at 100 nM was less effective and resulted in a 20% knockdown of miR-206 in myoblasts after 48 h.

5.4.4 CDC42 protein is up-regulated in response to miR-133a knockdown

Western analysis showed CDC42 was up-regulated in response to miR-133a and miR-206 knockdown providing evidence that CDC42 is a valid miR-133a and miR-206 target. CDC42 is a positive regulator of insulin signaling and cellular glucose uptake. CDC42 mediates insulin-stimulated GLUT4 translocation and glucose transport in a PI3-kinase dependent manner and acts upstream of PKC (Usui et al. 2003). CDC42 associates with p85 in response to insulin. Therefore, down-regulation of miR-133a and miR-206 in Type 2 diabetes skeletal muscle may be associated with compensatory actions on targets such as CDC42 to increase glucose uptake post-transcriptionally. However, it is unknown whether CDC42 protein levels are altered in the skeletal muscle of Type 2 diabetes patients *in-vivo* (Sundsten & Ortsäter, 2009) and muscle glucose uptake measurement was not possible in the present study.

CDC42 has not been previously associated with cancer cachexia pathogenesis, but it has been linked to muscle wasting. In a rat disuse model CDC42 protein was decreased 60% after 3 days of hind limb suspension and increased 172% following 12 h reload (Chockalingam et al. 2002). In addition, administration of a retroviral dominant-negative CDC42 vector caused skeletal muscle fibre atrophy in rats (Chockalingam et al. 2002). Activation of CDC42 in adult mice has been reported to significantly shorten life span, resulting in premature aging and muscle atrophy (Wang et al. 2007). In embryonic fibroblasts activation of CDC42 was linked to increased basal apoptosis through elevated c-JNK signaling (Wang et al. 2005). Apoptosis has been shown in skeletal muscle from gastro-intestinal cancer patients, indicated by increased DNA fragmentation (Busquets et al. 2007). Taken together these studies suggest decreased miR-133a suppression of CDC42 may cause increased apoptosis, which has been reported in cancer cachexia, but CDC42 has not yet been measured in skeletal muscle from cancer cachexia patients.

5.4.5 PTBP1 protein is up-regulated by miR-133a knockdown

In agreement with a previous study (Boutz et al. 2007) western analysis of PTBP1 following miR-133a knockdown showed PTBP1 protein was increased in skeletal muscle myoblasts. PTBP1 is a polypyrimidine tract binding protein, which belongs to the subfamily of ubiquitously expressed heterogeneous ribonucleoproteins (hnRNPs). PTBP1 is reportedly repressed during myoblast differentiation by miR-133 and miR-206 (Boutz et al. 2007). A luciferase reporter with the 3'UTR of PTB was repressed by miR-133 (Boutz et al. 2007). Transfection of ASOs to block miR-133 and miR-206 in myoblasts decreased expression of PTB dependent exons (Boutz et al. 2007) as PTBP1 contains an RNA recognition motif, which binds RNA and modulates pre-mRNA splicing via the ubiquitin-proteosome pathway. A recent proteomics study suggests mRNA-binding proteins in insulinoma cells respond to changes in glucose and proteins with PTBP1 bindings are modulated in response to glucose (Süss et al. 2009). Type 2 diabetes is associated with hyperglycaemia, but it is unknown whether mRNA-binding proteins change in skeletal muscle in response to glucose. PTBP1 has not been previously associated with changes in skeletal muscle from Type 2 diabetes or cancer cachexia patients.

5.4.6 No evidence of miR-133a or miR-206 regulation of SMEK2 or TGIF2

SMEK2 and TGIF2 appear not to be miR-133a or miR-206 targets in skeletal muscle myoblasts. In response to miR-133a and miR-206 knockdown SMEK2 and TGIF2 protein expression both appeared to be decreased or unchanged. Past research indicates microRNAs usually suppress targets, but more recently microRNAs have been reported to activate target proteins (Bartel, 2009; Vasudevan et al. 2007). SMEK2 is a suppressor of MEK, which has not been previously linked to Type 2 diabetes or cancer cachexia. However, it may have powerful functions via suppression of MEK, which is an important MAPK protein associated with multiple cellular pathways including insulin signaling. TGIF2 is a transcriptional repressor, which can directly bind DNA or interact with SMAD proteins leading to repression of TGF-β responsive transcription (Melhuish et al. 2001). Alternatively, the fluctuations in SMEK2 and TGIF2 protein levels after miR-133a and miR-206 knockdown may have been indirect. It was not possible in the present study to establish a direct interaction between miR-133a/206 and target 3'UTRs, as luciferase reporter assays were not possible in the present study.

5.4.7 Knockdown of miR-133a and miR-206 impacts other microRNAs

A problem with using mature microRNA ASOs to knockdown endogenous microRNAs is that close family members can also be affected. Both miR-133a and miR-206 have close family members in miR-133b and miR-1. The knockdown of miR-133a using the mature microRNA ASO approach resulted in ~40% knockdown of mature miR-206, ~30% knockdown of mature miR-1 and 70% knockdown of mature miR-133b. Knockdown of miR-206 using the mature microRNA ASO approach resulted in no effect on mature miR-133a, while mature miR-133b was increased ~30% and miR-1 was reduced ~30%. However, when miR-133a and miR-206 were transfected together, mature miR-1 and miR-206 was reduced by ~80%, while miR-133b was reduced by 50%. The effects of transfecting miR-133a ASOs on miR-133b in myoblasts could be explained by imperfect base pairing to the miR-133a ASO as mature miR-133a and miR-133b differ by only one nucleotide. Indeed, the effects of transfecting miR-206 ASOs on miR-1 could also be due to sequence complementarity in agreement with reports of widespread off-target effects of siRNAs mediated by sequence complementarity (Jackson et al. 2006). However, this cannot explain why miR-206 and miR-1 are knocked down by transfection of the miR-133a ASO in myoblasts. The current data suggests there must be some feedback from knockdown of mature miR-

133a on miR-206 and miR-1 therefore the effect of transfecting miR-133a and miR-206 ASOs into myoblasts on pri-microRNA transcription was examined.

5.4.8 Knockdown of miR-133a and miR-206 impacts microRNA transcription

Transfection of the miR-133a ASOs into myoblasts did not significantly affect pri-microRNA expression. In contrast, transfection of the miR-206 ASOs in myoblasts resulted in an >100% increase in pri-miR-1-2. Taken together these findings indicate there was surprisingly an effect of miR-206 knockdown on primary transcription of miR-1.

The mechanism regulating the effect of miR-206 on primary transcription of miR-1 was not established in this study. Previously, a feedback loop regulating miR-1 expression has been reported, as miR-1 targets MEF2 in myoblasts and MEF2 binding sites have been found on pri-miR-1-1 and pri-miR-1-2 (Liu et al. 2007; Rao et al. 2006). Furthermore, miR-206 inhibits MyoD expression and MyoD binding sites have been found in pri-miR-1-1, pri-miR-1-2, pri-miR-133a-1 and pri-miR-133a-2 (Rao et al. 2006). Therefore, down-regulation of miR-206 either directly or indirectly using the mature microRNA ASO approach may influence the transcription of miR-1 and miR-133a via de-repression of MyoD inhibitor (Kim et al. 2006). Nevertheless, the mature microRNA ASO approach remains the most commonly used method for knockdown of microRNAs in cell culture for experimental validation of targets (Horwich & Zamore, 2008; Carè et al. 2007).

5.4.9 Limitations

The experimental validation of targets is important when determining the function of microRNAs. Western blot is most often used for measuring protein abundance of targets. However, it is only practical to validate 1-2 targets using this method. Therefore it is not possible to confirm the actual number of genes targeted by one microRNA. The microRNA predictions used in this Chapter were based on the TargetScan algorithm. Estimates of false positive target predictions are around 40%, which is lower than other microRNA target prediction algorithms. However, even determining a false positive target prediction percentage is difficult as the complete set of microRNA targets is unknown. In the present study CDC42 and PTBP1 were up-regulated in response to miR-133a knockdown in murine skeletal muscle myoblasts. Previous studies often provide additional evidence of binding of microRNA to target

based on a 3'UTR luciferase assay. This would have been useful to check whether the predicted miR-133a binding sites in the 3'UTRs of SMEK2 and TGIF2 were functional, as SMEK2 and TGIF2 were not found to be up-regulated in response to miR-133a or miR-206 knockdown. A more general limitation of using a murine myoblast cell line is that it does not represent mature human skeletal muscle. Although there is ~90% homology between the mouse and human genomes the murine myoblasts may lack unknown contextual determinants that are present in adult muscle. For example, C2C12 myotubes appear not to require GLUT4 for cellular glucose uptake, whilst GLUT4 has been identified as the primary skeletal muscle glucose transporter in humans.

The knockdown of mature microRNA was attempted using two methods. The most widely used method involves transfection of mature microRNA ASOs into cells. However, in the present study the specificity of the ASO targeting miR-133a was not ideal. There were clearly effects on miR-1, miR-133b, miR-206 and potentially an unknown number of other microRNAs. Primary miR-133a and miR-1 transcripts were almost universally increased in response to mature miR-133 and miR-206 knockdown. Therefore caution is needed when interpreting the results of any microRNA knockdown experiments.

The experiments determining the effect of insulin on miR-1, miR-133a and miR-206 expression would have benefited from additional experiments being performed to increase power for statistical analysis. The use of a high insulin dose may be criticised for being unphysiological and results from these experiments should be treated with caution. Further experiments were conducted using a lower insulin dose, but the results were far from clear. In future it would be worthwhile to manipulate glucose independently of insulin to determine the effects of hypoglycemia on microRNA expression in myotubes. Further experiments using different concentrations of TNFα would be useful to determine whether miR-1, miR-133a and miR-206 changes are dose-dependent.

5.4.10 Future research directions

The experimental validation of all predicted miR-133a and miR-206 targets *in-vitro* would be useful to indicate how effective current microRNA target prediction algorithms are. Furthermore, in addition to validating microRNA targets it would be useful to establish whether miR-133a or miR-206 knockdown has functional

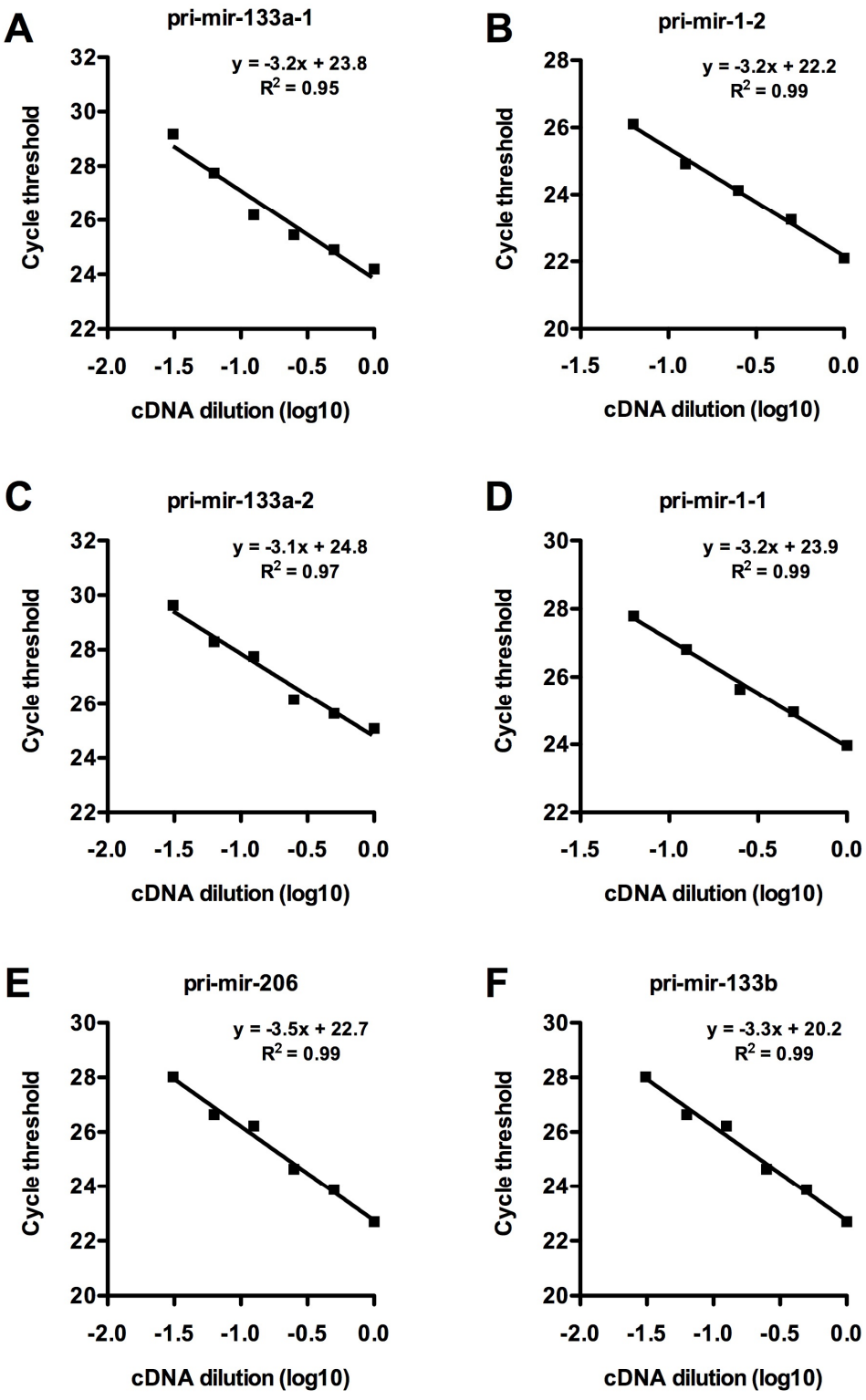
consequences on muscle glucose uptake or muscle protein degradation pathways, which are associated with Type 2 diabetes and cancer cachexia pathogenesis respectively.

It would be advantageous to conduct microRNA knockdown experiments on human primary skeletal muscle cultures rather than mouse myoblasts. Knockdown of microRNAs in human primary skeletal muscle cultures to validate targets would include contextual features, which may not be present in mouse myoblasts. The pri-microRNA and mature microRNA knockdown approaches appear to be effective in myoblasts and could be further optimised for knockdown of miR-133a and miR-206 in human primary skeletal muscle cell cultures. It will be important to establish that the miR-133a and miR-206 targets validated *in-vitro* in myoblasts are also up-regulated *in-vivo* in skeletal muscle of Type 2 diabetes and pancreatic cancer cachexia patients.

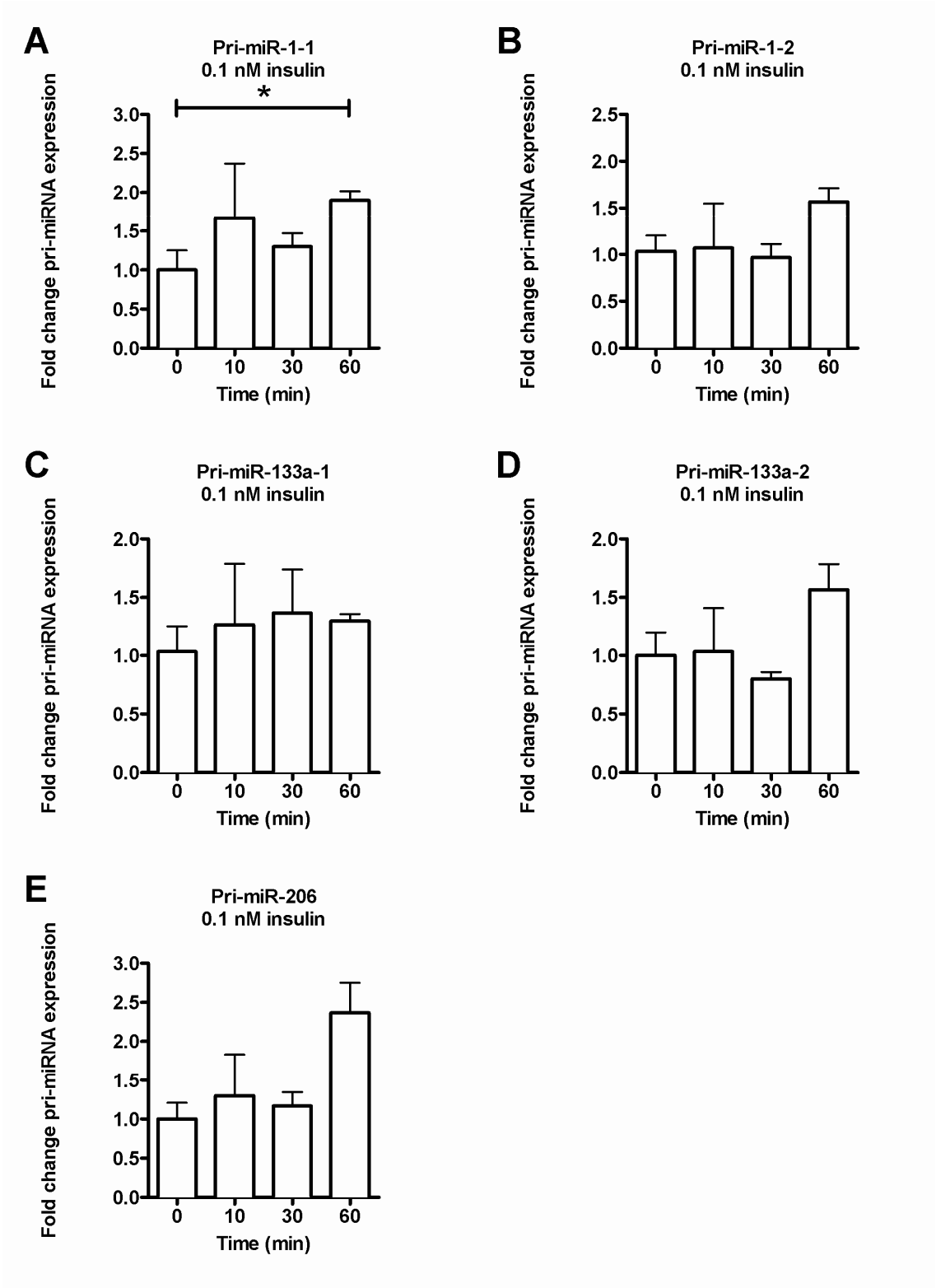
5.4.11 Conclusions

- CDC42 and PTBP1 protein levels in skeletal muscle myoblasts were elevated in response to miR-133a knockdown. However, SMEK2 and TGIF2 appeared not to be regulated by miR-133a or miR-206.
- Transfection of miR-133a and miR-206 ASOs in skeletal muscle myoblasts may lead to indirect effects on primary and mature miR-1, miR-133a and miR-206 expression.
- Specific knockdown of mature miR-133a and miR-206 is possible with ASOs targeting pri-microRNA transcripts in skeletal muscle myoblasts and could be useful to specifically target microRNAs with close family members.
- Mature miR-1, miR-133a and miR-206 expression in skeletal muscle myotubes may be regulated by insulin and TNFα, although the mechanism is unknown.

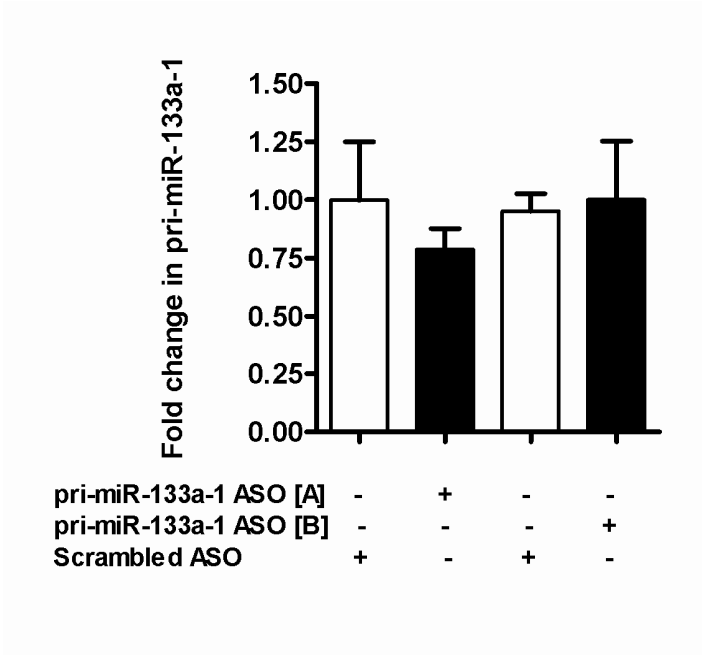
5.5. Supplementary data



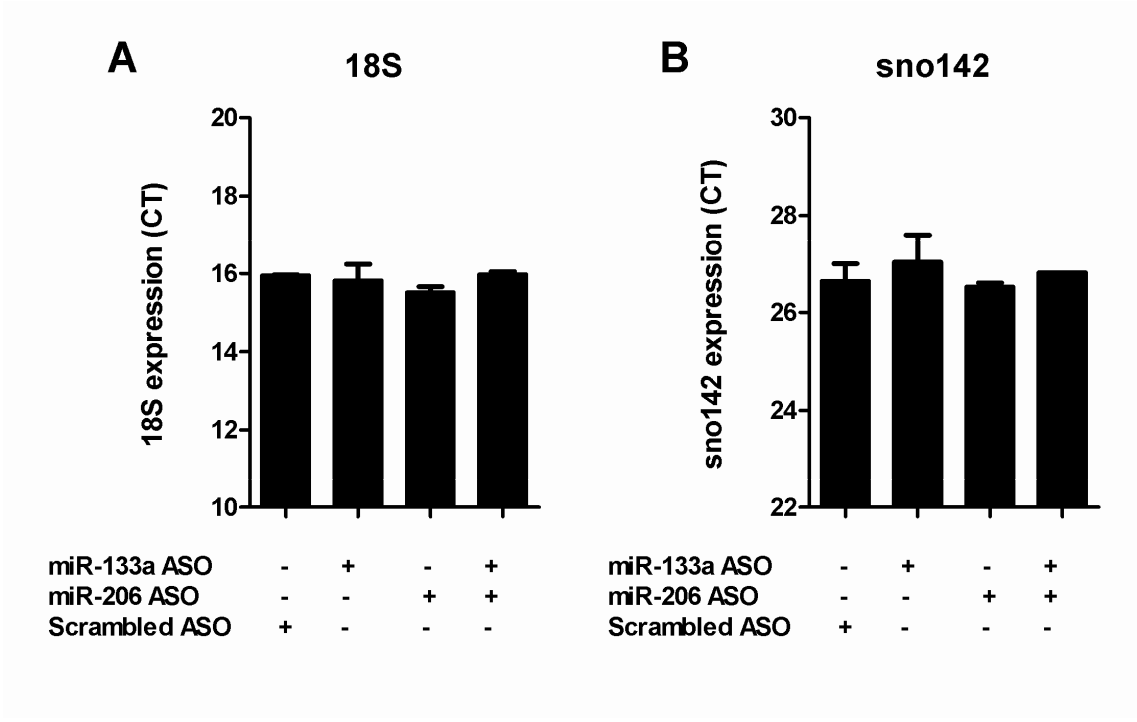
Supplementary Figure 5-1. Efficiency of pri-microRNA primers. (A) Pri-miR-133a-1, (B) Pri-miR-1-2, (C) Pri-miR-133a-2, (D) Pri-miR-1-1, (E) Pri-miR-206 and (F) Pri-miR-133b cycle thresholds across a range of cDNA dilutions.



Supplementary Figure 5-2 Fold change in (A) pri-miR-1-1, (B) pri-miR-1-2, (C) pri-miR-133a-1, (D) pri-miR-133a-2 and (E) pri-miR-206 in myotubes in response to 0.1 nM insulin treatment (n = 3). Data shown as mean ± SE.



Supplementary Figure 5-3. Pri-microRNA ASOs designed to target pri-miR-133a-1 were ineffective when transfected at 100 nM in myoblasts (n = 6). Data shown as mean ± SE.

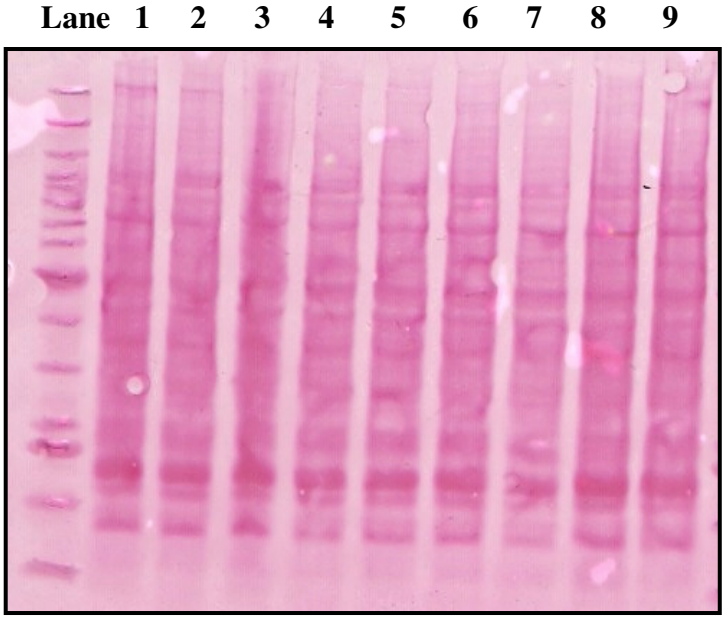


Supplementary Figure 5-4. (A) Stable 18S and (B) sno142 expression in myoblasts following mature microRNA ASO transfections. Data shown as mean ± SE.

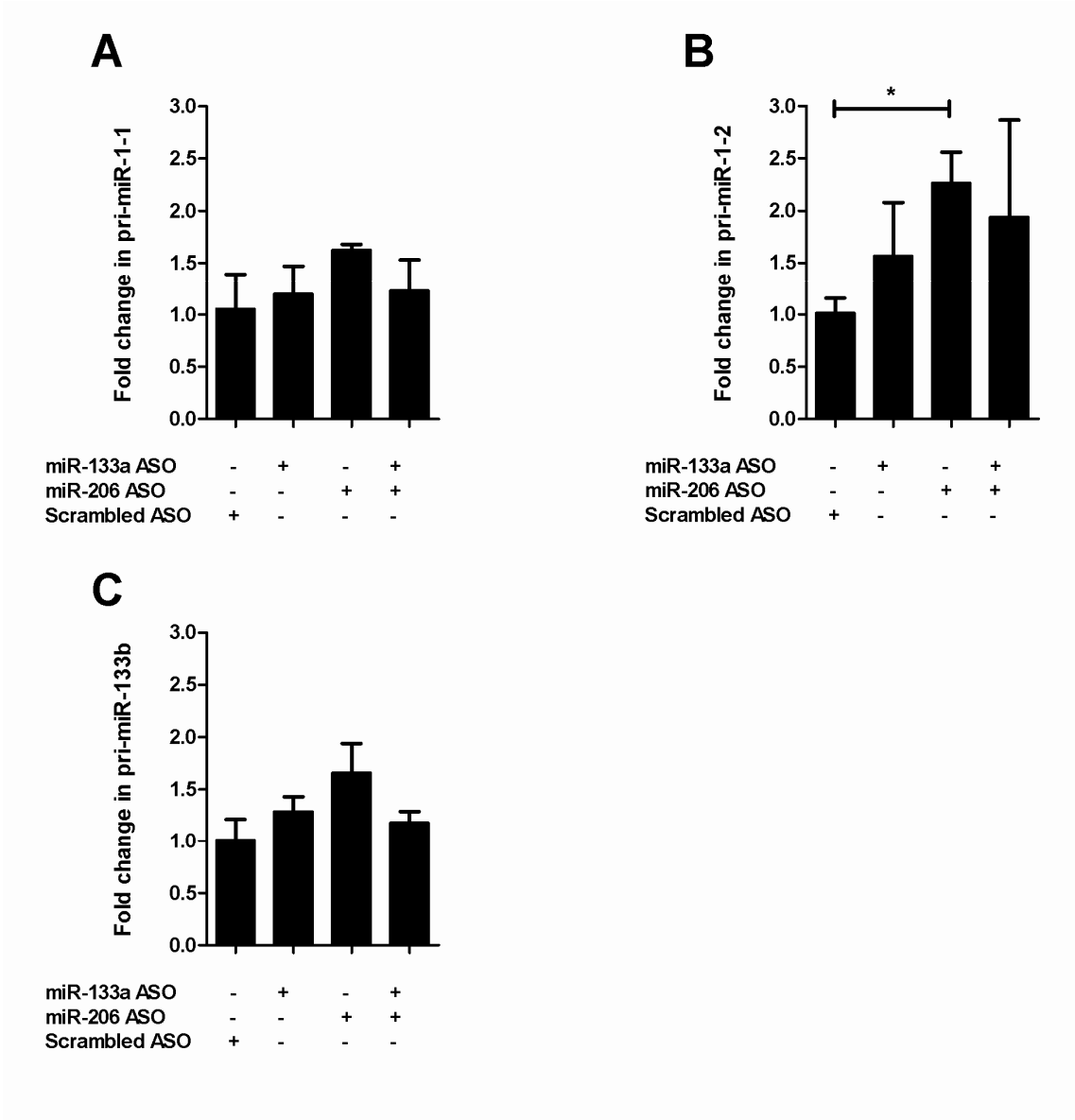
Supplementary Table 5-1. Total protein intensity for normalisation of Western blots determined from Ponceau Red stained nitrocellulose membrane

Sample	Lane	Total protein intensity*
miR-133a ASO	1	45344
miR-133a ASO	2	38228
miR-206 ASO	3	40988
miR-206 ASO	4	31861
miR-133a + 206 ASO	5	36324
miR-133a + 206 ASO	6	39089
Scrambled ASO	7	33432
Scrambled ASO	8	43065
Non-transfected	9	42900

* Determined using ImageJ software (NIH).



Supplementary Figure 5-5. Total protein from Ponceau Red stained nitrocellulose membrane.



Supplementary Figure 5-6. Effect of miR-133a and miR-206 knockdown on transcription of (A) pri-miR-1-1, (B) pri-miR-1-2 and (C) pri-miR-133b. Data from duplicate transfections shown as mean ± SE. * *P* < 0.05

Chapter 6 - Final Conclusions

In recent years evidence that microRNAs are important global regulators of gene expression and biomarkers of disease has been accumulating. This thesis focused on identifying microRNA biomarkers of cancer cachexia and Type 2 diabetes in skeletal muscle. There has been no previous research on microRNA expression in skeletal muscle from Type 2 diabetes or cancer cachexia patients, thus this thesis presents some very novel data.

6.1. Novel microRNA biomarkers in cancer cachexia patients

In Chapter 3, muscle specific miR-1, miR-133a, miR-133b and miR-206 expression were shown to be negatively related to weight loss in pancreatic cancer cachexia patients. Weight loss in cancer cachexia patients is a commonly used indicator of cancer cachexia severity. Therefore, muscle specific miR-1, miR-133a, miR-133b and miR-206 may be potential biomarkers of cancer cachexia severity in pancreatic cancer patients. Past studies have established the importance of these muscle specific microRNAs in myogenesis and muscle growth (Kim et al. 2006; Nakajima et al. 2006; Chen et al. 2006; Boutz et al. 2007; van Rooij et al. 2008), thus the down-regulation of these microRNAs in pancreatic cancer cachexia patients observed in Chapter 3 is consistent with the role of these microRNAs in muscle growth and differentiation.

However, an important caveat to bear in mind is miR-1, miR-133a, miR-133b and miR-206 expression was not significantly related to weight loss when the cancer patients with different tumour types were considered. The prevalence of cachexia has been reported to be higher among pancreatic cancer patients compared to other cancer patients but the mechanism remains unexplained (Fearon, 1992). No universal cause for cachexia has been identified, which suggests for some cancer patients these microRNAs may be biomarkers of cachexia pathogenesis, but for others these microRNAs may be less important. Gene ontology enrichment analysis suggests miR-1, miR-133a, miR-133b and miR-206 may regulate transcriptional regulators thus may have widespread indirect effects on gene expression, but the possible functional role of these microRNAs in cancer cachexia was not clear.

During skeletal muscle differentiation miR-1, miR-133a and miR-206 are strongly induced. Therefore down-regulation of these microRNAs may indicate proliferation

and stem-cell renewal is occurring (Boutz et al. 2007; Rao et al. 2006). Previously, it has been suggested cachexia may stimulate an increase in stem cells populations in an attempt to maintain muscle mass (Berardi et al. 2008). For example, increases in hematopoietic stem cells have been reported in skeletal muscle of C-26 tumour bearing mice, thus supporting the idea that muscle regeneration may be occurring during cachexia (Berardi et al. 2008).

It was not possible to determine whether miR-1, miR-133a, miR-133b and miR-206 were directly involved in cachexia pathogenesis or were only biomarkers of cancer cachexia severity due to the cross-sectional study design. In future, a longitudinal study of microRNA changes in cancer cachexia patients would help establish whether these microRNAs contribute to cancer cachexia pathogenesis. There was no evidence of differences in microRNA processing genes between control, pre-cachexia and cachexia patients, in agreement with an earlier study on muscle atrophy in mice (McCarthy et al. 2007). However this does not preclude that post-transcriptional changes may influence microRNA processing genes in cancer cachexia patients (Wiesen & Tomasi, 2009).

A major limitation of the present cancer cachexia cohort was that cachexia severity was based on patients estimated body mass prior to cancer diagnosis compared to measured body mass during clinical examination before tumour resection. Patients may have over- or under-estimated initial body mass, which would have affected the determination of weight loss and cachexia severity. It is unknown how reliable patient estimates of body mass were and it was not possible to check medical records.

In addition, miR-21 was measured in cancer cachexia patients as a potential biomarker of cancer cachexia severity because miR-21 was found to be dysregulated in ICU patients characterised by severe inflammation and muscle wasting (Fredriksson et al. 2008). There was an indication miR-21 may be elevated in cancer cachexia patients similar to previous findings in patients with various muscular dystrophies, but these results were not significant (Eisenberg et al. 2007). Nevertheless, the predicted targets of miR-21 include genes involved in the ubiquitin-proteolysis pathway, which has been implicated in cancer cachexia (Williams et al. 1999). Further studies would be necessary in a larger cross-sectional cancer cachexia cohort to establish whether miR-21 may be a useful biomarker of cancer cachexia severity.

6.2. Down-regulated miR-133a and miR-206 in Type 2 diabetes patients

In skeletal muscle from Type 2 diabetes patients, miR-133a and miR-206 were found to be significantly down-regulated compared to age, BMI and VO_2max matched controls with normal glucose tolerance. In addition, miR-133a expression alone could explain 30-40% variance in glucose tolerance and fasting glucose both part of the WHO criteria for diagnosis of impaired glucose tolerance and Type 2 diabetes (WHO, 2006). Furthermore, miR-133a and miR-206 expression could predict 30% of the variance in HbA1c which indicates prolonged hyperglycaemia and HOMA[IR] which indicates insulin resistance. Therefore, miR-133a may be a potential biomarker for the development of insulin resistance and Type 2 diabetes. However, it was not possible to determine whether miR-133a expression changes were a cause or effect of pathophysiological changes leading to Type 2 diabetes.

Discovery of new biomarkers is important as earlier diagnosis of Type 2 diabetes would help to significantly improve morbidity and mortality rates (Roglic et al. 2005; Shaw et al. 2000; Wild et al. 2004; WHO, 2006; Zimmet et al. 2001). MicroRNA analysis requires only small amounts of RNA using RT-qPCR (Cissell & Deo, 2009; Chen et al. 2005), but muscle biopsies are not useful for routine clinical testing. Thus it would be useful to determine whether similar changes in microRNA expression occur in blood. In the future it will be important to establish in a larger population sample how sensitive microRNA expression is to early changes in glucose tolerance indicators and furthermore whether microRNA expression can predict the development of Type 2 diabetes.

Transcription of miR-133a appeared to be unaltered in Type 2 diabetes patients and there was no change in the expression of the primary microRNA processing genes DROSHA and DGCR8. These findings suggest a factor downstream of primary microRNA transcription appears to be responsible for the down-regulation of mature miR-133a and miR-206 expression in Type 2 diabetes. Pre-miR-133a appeared to be rapidly processed into mature miR-133a in agreement with previous findings (Lee et al. 2008) and the pre-microRNA processing genes DICER and AGO2 were unaltered in Type 2 diabetes. Taken together these findings suggest there must be other regulatory steps after initial microRNA transcription that determines mature microRNA levels. Recent studies suggest an important role of RNA-binding proteins in regulating

microRNA processing (Guil & Cáceres, 2007; Kim et al. 2009; Michlewski et al. 2008) or alternatively perhaps microRNA degradation is regulated in Type 2 diabetes. Further studies would be useful to identify the factors which cause post-transcriptional down-regulation of miR-133a in Type 2 diabetes. No studies have yet considered when or how microRNAs are degraded and this would be worthwhile to establish as this may explain why mature miR-133a is lower in skeletal muscle from Type 2 diabetes patients.

In Chapter 4, analysis of existing microarray data after miR-133a overexpression in HeLa cells (Grimson et al. 2007) revealed multiple down-regulated miR-133a targets have been previously linked to diabetes, including CDC42. However, in Type 2 diabetes patients there was evidence of increased miR-206 target expression, but not miR-133a target expression. Therefore, miR-133a may act primarily via translational repression of targets. In the present study it was not possible to determine whether miR-133a or miR-206 target proteins were increased *in-vivo* in skeletal muscle from Type 2 diabetes patients. Gene ontology enrichment analysis (Sherman et al. 2007) revealed the functional consequences of miR-133a in adult skeletal muscle may be partly through repression of intracellular transport proteins and protein phosphatases. Insulin signaling and hence glucose uptake are known to be inhibited by protein phosphatases (Muoio & Newgard, 2008; Youngren et al. 2007), but these were not measured in Type 2 diabetes patients in the present study. In future, *in-vivo* measurement of microRNA target proteins in skeletal muscle from Type 2 diabetes patients may help to reveal the importance of post-transcriptional regulation by microRNAs.

In agreement with published microarray data from diabetic rats (He et al. 2007), miR-29a was found to be up-regulated in skeletal muscle from Type 2 diabetes patients. Furthermore, overexpression of miR-29a is reported to suppress insulin-stimulated glucose uptake in adipocytes (He et al. 2007). Hyperglycaemia and hyperinsulinaemia were both reported to mimic miR-29 suppression of insulin-stimulated glucose uptake (He et al. 2007). However, recent microRNA array data from diabetic GK rats failed to show miR-29a was up-regulated (Huang et al. 2009). The lack of concordance between two microRNA array studies conducted in diabetic GK rats may have been due to the use of different custom microarray platforms (Huang et al. 2009; He et al. 2007). A recent study compared five commercial microRNA array platforms for agreement and reported a lack of agreement between some platforms (Sato et al. 2009). Despite the

limitations in previous studies on microRNAs in diabetic GK rats, most importantly in human skeletal muscle miR-29a is up-regulated *in-vivo* in Type 2 diabetes patients. Therefore, miR-29a appears to be another potential microRNA biomarker of human Type 2 diabetes. In future studies, it would be worthwhile determining whether miR-29a transcription or processing is altered in human Type 2 diabetes.

The potential importance of post-transcriptional regulation in skeletal muscle in Type 2 diabetes has been supported by recent genome-wide transcriptome studies which found no evidence of transcriptional changes in skeletal muscle from Type 2 diabetes patients when compared to BMI and physical activity matched controls (J. Timmons, personal communication). Importantly, in the present study Type 2 diabetes patients and controls were matched for BMI and physical activity as these factors are known to influence gene expression (Krämer et al. 2006; Ling et al. 2004; Mathai et al. 2008; Sriwijitkamol et al. 2007; Timmons et al. 2006). In Chapter 2, studies demonstrating microRNAs target insulin signaling proteins were reviewed (Gauthier & Wollheim, 2006; Hennessy & O'Driscoll, 2008; Poy et al. 2007; Tang et al. 2008), but most of these studies were not performed in human skeletal muscle. Nevertheless it does highlight there are many more microRNAs which may be involved in the development of Type 2 diabetes which remain to be verified in human skeletal muscle. Taken together the present findings and past studies provide strong evidence to suggest microRNAs are not only novel biomarkers of insulin resistance and Type 2 diabetes, but may also play a role in Type 2 diabetes pathogenesis. However, a larger longitudinal study would be needed to establish whether microRNAs are down-regulated in skeletal muscle as individuals develop Type 2 diabetes. Alternatively, microRNA changes in Type 2 diabetes could be secondary effects of hyperglycaemia and hyperinsulinaemia due to insulin resistance and pancreatic β-cell dysfunction.

6.3. MicroRNAs respond to extracellular factors in myotubes

In Chapter 5, to determine possible regulators of microRNA expression in Type 2 diabetes and cancer cachexia the effects of extracellular factors on microRNA expression in myotubes was determined. Hyperglycaemia and hyperinsulinaemia are characteristics of Type 2 diabetes and develop with insulin resistance and pancreatic β-cell dysfunction. Here it was only possible to examine the response of miR-1, miR-133a and miR-206 to hyperinsulinaemia, due to RNA degradation occurring in cell samples subjected to high or low glucose. Nevertheless, in response to insulin miR-1

appeared to increase, miR-133a appeared to decrease, while miR-206 remained stable in myotubes. These findings support the idea that the down-regulation of miR-133a observed in Type 2 diabetes patients may in part be due to the effects of hyperinsulinaemia, although it would be worthwhile repeating this experiment over a longer time period. There were also transcriptional changes associated with insulin treatment in myotubes. Insulin treatment increased expression of pri-miR-1-1, pri-miR-133a-1 and pri-miR-206 but had no affect on pri-miR-1-2, pri-miR-133a-2 or pri-miR-133b. The lack of concordance between primary and mature microRNA expression in response to insulin suggests further regulation occurs in the microRNA biogenesis and processing pathway, which determines functional mature microRNA expression. However, it was not possible in the present study to examine the mechanism by which insulin influences primary and mature miR-1, miR-133a and miR-206 expression. In future studies, it would be worthwhile to determine whether skeletal muscle microRNA expression changes in response to experimental hyperglycaemia or hyperinsulinaemia in humans.

In Type 2 diabetes patients elevated plasma TNFα is reported, which may impair insulin signaling and hence muscle glucose uptake (Lindmark et al. 2006; Plomgaard et al. 2007). In addition, TNFα is known to trigger protein breakdown in skeletal muscle, thus has been implicated in the pathogenesis of cancer cachexia (Tisdale 2005; Stephens et al. 2008; Seruga et al. 2008). Treatment of myotubes with TNFα led to a decrease in miR-1, miR-133a and miR-206 expression after 1 h and after 24 h. These findings suggest TNFα may be a regulator of muscle specific microRNA expression; this is plausible as TNFα has recently been reported to modulate microRNA expression in adipocytes (Xie et al. 2009). Further studies would be useful to verify muscle specific microRNA expression responds to TNFα, for example by reducing TNFα levels in sepsis patients using TNFα antibodies.

6.4. MicroRNA targets regulated in myoblasts

Knockdown of miR-133a and miR-206 showed CDC42 protein was up-regulated confirming CDC42 is regulated by miR-133a and miR-206 in skeletal muscle myoblasts. CDC42 mediates insulin-stimulated GLUT4 translocation and glucose transport in a PI3-kinase dependent manner (Usui et al. 2003). Therefore, down-regulation of miR-133a and miR-206 in Type 2 diabetes skeletal muscle may be

associated with compensatory actions on targets such as CDC42 to increase glucose uptake. However, it is unknown whether CDC42 protein levels are altered in the skeletal muscle of Type 2 diabetes patient's *in-vivo* (Sundsten & Ortsäter, 2009).

CDC42 has not been previously associated with cancer cachexia pathogenesis, but it has been linked to muscle wasting. For example, in a rat disuse model CDC42 protein was decreased 60% after 3 days of hind limb suspension, in addition administration of a retroviral dominant-negative CDC42 vector caused skeletal muscle fibre atrophy in rats (Chockalingam et al. 2002). Therefore, miR-133a and miR-206 regulation of CDC42 may be relevant in cancer cachexia, although it was not possible to measure CDC42 protein levels in cancer cachexia patients in the present study.

In agreement with a previous study western analysis of PTBP1 protein following miR-133a knockdown increased PTBP1 protein levels (Boutz et al. 2007). PTBP1 is a polypyrimidine tract binding protein, which belongs to the subfamily of ubiquitously expressed heterogeneous ribonucleoproteins (hnRNPs). A recent proteomics study suggests mRNA-binding proteins such as PTBP1 respond to changes in glucose in insulinoma cells (Süss et al. 2009). Type 2 diabetes is associated with hyperglycaemia, but it is unknown whether mRNA-binding proteins change in skeletal muscle in response to glucose. Findings from the present study establish miR-133a targets PTBP1, but it remains to be determined whether PTBP1 protein is elevated *in-vivo* in cancer cachexia or Type 2 diabetes patients.

6.5. Primary microRNA knockdown is possible in myoblasts

Typically, microRNA knockdown with anti-sense oligonucleotides (ASOs) is used to help experimentally validate predicted microRNA targets (Horwich & Zamore, 2008; Carè et al. 2007). In Chapter 5, there appeared to be indirect effects on other highly expressed muscle specific microRNAs when transfecting myoblasts with ASOs targeting mature microRNAs. Improved specificity to knockdown microRNAs can be achieved by transfecting myoblasts with ASOs targeting pri-microRNA transcripts. For example, in Chapter 5 knockdown of mature miR-133a was demonstrated in response to knockdown of pri-miR-133a-2 using ASOs. In addition, pri-miR-206 knockdown decreased mature miR-206 levels in myoblasts. In the present study the mechanism by which the pri-microRNA ASO was acting was not determined, but it has been suggested ASOs may act via an RNase H-based mechanism causing degradation of the target

transcript (Davis et al. 2006). Nevertheless, it appears targeting pri-microRNAs may be an effective strategy to knockdown mature microRNAs and may be useful to experimentally validate microRNA targets in the future.

6.6. Future research directions

Longitudinal studies are needed to determine whether microRNAs are sensitive to the early changes in skeletal muscle occurring in the pre-cachexia state (Bozzetti & Mariani, 2008) and pre-diabetes state (Edelstein et al. 1997; WHO, 2006) compared to other biomarkers such as fasting glucose, glucose tolerance, CRP and TNFα. It would be advantageous to use more sensitive indicators of cancer cachexia severity, for example muscle-mass loss determined by MRI or DEXA scanning. In addition, the hyperinsulinaemic euroglycaemic clamp would provide a more sensitive indicator of insulin resistance in Type 2 diabetes rather than the oral glucose tolerance test used here.

Furthermore, in addition to validating microRNA targets it would be useful to establish whether miR-133a or miR-206 knockdown has functional consequences on muscle glucose uptake or muscle protein degradation pathways, which are associated with Type 2 diabetes and cancer cachexia pathogenesis respectively. It would be advantageous to conduct microRNA knockdown experiments on human primary skeletal muscle cultures rather than mouse C2C12 myoblasts.

It will be important to establish that the miR-133a and miR-206 targets validated *in-vitro* in myoblasts are also up-regulated *in-vivo* in skeletal muscle of Type 2 diabetes and pancreatic cancer cachexia patients. From the perspective of microRNA function in cancer cachexia and Type 2 diabetes, new studies measuring global changes in microRNA targets at the mRNA and protein level in patient skeletal muscle would provide more robust evidence of the magnitude of microRNA regulation in disease pathogenesis.

6.7. Final word

There is now huge interest in the role of microRNAs in chronic human diseases including cancer, Type 2 diabetes, cardiovascular disease and muscular dystrophies (van Rooij et al. 2008; Chen et al. 2009; Yang & Wu, 2007; Couzin, 2008; Hennessy & O'Driscoll, 2008). MicroRNA target predictions suggest over 60% of protein coding genes have been under evolutionary pressure to maintain microRNA target sites

(Friedman et al. 2009). Furthermore, over 45,000 conserved microRNA target sites have now been identified in human 3'UTRs (Friedman et al. 2009). Therefore microRNAs have significant potential to influence diease pathogenesis.

This thesis demonstrates that skeletal muscle microRNAs may be involved in the pathogenesis of Type 2 diabetes and cancer cachexia. Furthermore microRNAs may provide early biomarkers of Type 2 diabetes and cancer cachexia thus facilitating earlier intervention. MicroRNA-133a was the strongest predictor of existing diagnostic criteria. However, this thesis is based on cross-sectional microRNA expression data, therefore further longitudinal studies would be required to determine whether microRNA expression in human skeletal muscle can predict Type 2 diabetes and cancer cachexia development.

Chapter 7 - Appendices

Appendix 7-1. GO Molecular function terms enriched among miR-1/206 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
NUCLEIC ACID BINDING	72	217	1974	9167	0.000	0.0%
TRANSCRIPTION REGULATOR ACTIVITY	43	217	1002	9167	0.000	0.0%
BINDING	184	217	6819	9167	0.000	0.0%
PROTEIN BINDING	105	217	3398	9167	0.000	0.0%
DNA BINDING	54	217	1482	9167	0.001	0.0%
TRANSCRIPTION FACTOR ACTIVITY	30	217	734	9167	0.004	0.0%
PHOSPHOPROTEIN PHOSPHATASE ACTIVITY	6	217	128	9167	0.184	144.0%
UBIQUITIN-PROTEIN LIGASE ACTIVITY	7	217	157	9167	0.166	147.0%

Appendix 7-2. GO Cellular component terms enriched among miR-1/206 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
MEMBRANE-BOUND ORGANELLE	126	202	3740	8448	0.000	0.0%
NUCLEUS	94	202	2482	8448	0.000	0.0%
INTRACELLULAR ORGANELLE	137	202	4330	8448	0.000	0.0%
CELL	195	202	7620	8448	0.001	0.0%
ENDOMEMBRANE SYSTEM	16	202	240	8448	0.001	0.0%
GOLGI STACK	15	202	260	8448	0.004	0.0%
GOLGI APPARATUS	17	202	332	8448	0.006	0.0%
ORGANELLE MEMBRANE	15	202	302	8448	0.013	9.1%

Appendix 7-3. GO Biological process terms enriched among miR-1/206 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
REGULATION OF CELLULAR PHYSIOLOGICAL PROCESS	86	214	2246	8892	0.000	0.0%
REGULATION OF CELLULAR METABOLISM	63	214	1551	8892	0.000	0.0%
REGULATION OF TRANSCRIPTION	57	214	1424	8892	0.000	0.0%
CELL ORGANIZATION AND BIOGENESIS	45	214	1120	8892	0.001	0.0%
VESICLE-MEDIATED TRANSPORT	17	214	263	8892	0.001	0.0%
PROTEIN LOCALIZATION	19	214	412	8892	0.010	0.0%
SECRETORY PATHWAY	9	214	132	8892	0.014	3.0%
ENZYME LINKED RECEPTOR PROTEIN SIGNALING PATHWAY	10	214	155	8892	0.012	3.3%
ESTABLISHMENT OF LOCALIZATION	64	214	2015	8892	0.011	3.6%
METABOLISM	137	214	5050	8892	0.020	5.6%
MUSCLE DEVELOPMENT	7	214	94	8892	0.025	7.5%
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE SIGNALING PATHWAY	8	214	117	8892	0.022	8.1%
NEGATIVE REGULATION OF CELLULAR PHYSIOLOGICAL PROCESS	21	214	529	8892	0.028	9.3%

Appendix 7-4. GO Molecular function terms enriched among miR-21 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
TRANSCRIPTION REGULATOR ACTIVITY	26	111	1033	9743	0.000	0.0%
DNA BINDING	31	111	1539	9743	0.001	0.0%
RNA BINDING	11	111	380	9743	0.011	0.0%
UBIQUITIN-PROTEIN LIGASE ACTIVITY	7	111	165	9743	0.011	0.0%
STRUCTURE-SPECIFIC DNA BINDING	4	111	41	9743	0.011	0.0%
LIGASE ACTIVITY, FORMING CARBON-NITROGEN BONDS	7	111	198	9743	0.024	11.1%

Appendix 7-5. GO Cellular component terms enriched among miR-21 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
NUCLEUS	50	102	2557	8941	0.000	0.0%
INTRACELLULAR MEMBRANE-BOUND ORGANELLE	58	102	3848	8941	0.004	0.0%
MEMBRANE-BOUND ORGANELLE	58	102	3850	8941	0.004	0.0%
INTRACELLULAR	74	102	5326	8941	0.005	0.0%
PROTEIN COMPLEX	25	102	1430	8941	0.027	14.3%
ORGANELLE	62	102	4463	8941	0.022	16.7%
INTRACELLULAR ORGANELLE	62	102	4461	8941	0.021	20.0%
UBIQUITIN LIGASE COMPLEX	5	102	120	8941	0.047	50.0%

Appendix 7-6. GO Biological process terms enriched among miR-21 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
REGULATION OF CELLULAR PROCESS	48	108	2425	9408	0.000	0.0%
REGULATION OF TRANSCRIPTION, DNA-DEPENDENT	32	108	1391	9408	0.000	0.0%
REGULATION OF PHYSIOLOGICAL PROCESS	46	108	2398	9408	0.000	0.0%
NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	40	108	2317	9408	0.004	0.0%
DNA PACKAGING	7	108	213	9408	0.034	11.8%
PROTEIN UBIQUITINATION	5	108	120	9408	0.048	19.0%

Appendix 7-7. GO Molecular function terms enriched among miR-23a targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
TRANSCRIPTION FACTOR ACTIVITY	54	355	764	9743	0.000	0.0%
DNA BINDING	89	355	1539	9743	0.000	0.0%
NUCLEIC ACID BINDING	111	355	2056	9743	0.000	0.0%
RNA POLYMERASE II TRANSCRIPTION FACTOR ACTIVITY	18	355	186	9743	0.000	0.0%
PROTEIN SERINE/THREONINE KINASE ACTIVITY	22	355	306	9743	0.004	0.0%
PROTEIN KINASE ACTIVITY	28	355	430	9743	0.004	0.0%
ION BINDING	110	355	2479	9743	0.012	7.1%
CALMODULIN BINDING	11	355	119	9743	0.011	8.3%

Appendix 7-8. GO Cellular component terms enriched among miR-23a targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
NUCLEUS	148	333	2557	8941	0.000	0.0%
MEMBRANE-BOUND ORGANELLE	184	333	3850	8941	0.000	0.0%
INTRACELLULAR ORGANELLE	205	333	4461	8941	0.000	0.0%
NUCLEAR ENVELOPE	8	333	74	8941	0.020	37.5%
GOLGI APPARATUS	21	333	350	8941	0.036	70.0%
MUSCLE MYOSIN	3	333	14	8941	0.093	193.0%

Appendix 7-9. GO Biological process terms enriched among miR-23a targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
REGULATION OF TRANSCRIPTION	89	344	1472	9408	0.00	0.0%
TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER	32	344	423	9408	0.00	0.0%
POSITIVE REGULATION OF BIOLOGICAL PROCESS	34	344	501	9408	0.00	0.0%
NEGATIVE REGULATION OF TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER	9	344	62	9408	0.00	0.0%
CELL ADHESION	34	344	548	9408	0.00	0.0%
WNT RECEPTOR SIGNALING PATHWAY	10	344	85	9408	0.00	0.0%
CELL PROLIFERATION	29	344	473	9408	0.01	0.0%
PROTEIN AMINO ACID PHOSPHORYLATION	26	344	427	9408	0.01	2.9%
REGULATION OF CELL CYCLE	24	344	376	9408	0.01	2.9%
NEGATIVE REGULATION OF PROGRESSION THROUGH CELL CYCLE	11	344	126	9408	0.02	5.4%
PROTEIN MODIFICATION	51	344	1036	9408	0.02	7.1%

Appendix 7-10. GO Molecular function terms enriched among miR-27b targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
PROTEIN SERINE/THREONINE KINASE ACTIVITY	39	435	306	9743	0.000	0.0%
TRANSCRIPTION REGULATOR ACTIVITY	80	435	1033	9743	0.000	0.0%
PROTEIN-TYROSINE KINASE ACTIVITY	23	435	168	9743	0.000	0.0%
CAMP-DEPENDENT PROTEIN KINASE ACTIVITY	23	435	172	9743	0.000	0.0%
PROTEIN KINASE CK2 ACTIVITY	22	435	168	9743	0.000	0.0%
TRANSFERASE ACTIVITY, TRANSFERRING PHOSPHORUS-CONTAINING GROUPS	55	435	688	9743	0.000	0.0%
ATP BINDING	66	435	916	9743	0.000	0.0%
ADENYL NUCLEOTIDE BINDING	67	435	945	9743	0.000	0.0%
PROTEIN BINDING	189	435	3453	9743	0.000	0.0%
PHOSPHOPROTEIN PHOSPHATASE ACTIVITY	16	435	131	9743	0.001	0.0%
PHOSPHORIC ESTER HYDROLASE ACTIVITY	22	435	243	9743	0.003	0.0%
STEROID HORMONE RECEPTOR ACTIVITY	8	435	50	9743	0.006	0.0%
STRUCTURAL CONSTITUENT OF CYTOSKELETON	10	435	81	9743	0.010	3.1%
LIGAND-DEPENDENT NUCLEAR RECEPTOR ACTIVITY	8	435	53	9743	0.009	3.2%
PROTEIN BINDING, BRIDGING	8	435	60	9743	0.017	5.9%
RNA POLYMERASE II TRANSCRIPTION FACTOR ACTIVITY	16	435	186	9743	0.019	8.1%
TRANSCRIPTION COFACTOR ACTIVITY	18	435	219	9743	0.018	8.3%

Appendix 7-11. GO Cellular component terms enriched among miR-27b targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
NUCLEUS	164	397	2557	8941	0.000	0.0%
MEMBRANE-BOUND ORGANELLE	206	397	3850	8941	0.000	0.0%
INTRACELLULAR	268	397	5326	8941	0.001	0.0%
INTRACELLULAR ORGANELLE	229	397	4461	8941	0.001	0.0%

Appendix 7-12. GO Biological process terms enriched among miR-27b targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
DEVELOPMENT	117	426	1598	9408	0.000	0.0%
PROTEIN MODIFICATION	82	426	1036	9408	0.000	0.0%
PROTEIN AMINO ACID PHOSPHORYLATION	44	426	427	9408	0.000	0.0%
REGULATION OF TRANSCRIPTION	101	426	1472	9408	0.000	0.0%
PHOSPHORYLATION	46	426	522	9408	0.000	0.0%
METABOLISM	273	426	5213	9408	0.000	0.0%
LOCALIZATION OF CELL	23	426	213	9408	0.000	0.0%
CELL DIFFERENTIATION	35	426	431	9408	0.001	0.0%
POSITIVE REGULATION OF TRANSCRIPTION, DNA-DEPENDENT	9	426	67	9408	0.010	2.3%
ENZYME LINKED RECEPTOR PROTEIN SIGNALING PATHWAY	15	426	156	9408	0.011	2.2%
INTRACELLULAR SIGNALING CASCADE	52	426	824	9408	0.012	2.1%
WNT RECEPTOR SIGNALING PATHWAY	10	426	85	9408	0.014	4.1%
TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER	30	426	423	9408	0.016	3.9%
SMALL GTPASE MEDIATED SIGNAL TRANSDUCTION	15	426	177	9408	0.029	10.2%
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE SIGNALING PATHWAY	11	426	117	9408	0.039	14.8%
UBIQUITIN CYCLE	19	426	256	9408	0.042	14.5%

Appendix 7-13. GO Molecular function terms enriched among miR-29 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
EXTRACELLULAR MATRIX STRUCTURAL CONSTITUENT	21	372	88	9743	0.000	0.0%
BINDING	306	372	7066	9743	0.000	0.0%
PROTEIN BINDING	168	372	3453	9743	0.000	0.0%
CATION BINDING	116	372	2282	9743	0.000	0.0%
STRUCTURAL MOLECULE ACTIVITY	41	372	605	9743	0.000	0.0%
CALCIUM ION BINDING	45	372	696	9743	0.001	0.0%
TRANSCRIPTION REGULATOR ACTIVITY	59	372	1033	9743	0.001	0.0%
ION BINDING	120	372	2479	9743	0.002	0.0%
METALLOENDOPEPTIDASE ACTIVITY	11	372	93	9743	0.003	0.0%
DNA BINDING	79	372	1539	9743	0.004	0.0%
TRANSCRIPTION FACTOR ACTIVITY	44	372	764	9743	0.006	0.0%
PROTEIN SERINE/THREONINE PHOSPHATASE ACTIVITY	7	372	56	9743	0.019	15.4%

Appendix 7-14. GO Cellular component terms enriched among miR-29 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
COLLAGEN	19	342	33	8941	0.000	0.0%
EXTRACELLULAR MATRIX	38	342	241	8941	0.000	0.0%
FIBRILLAR COLLAGEN	8	342	10	8941	0.000	0.0%
COLLAGEN TYPE IV	5	342	6	8941	0.000	0.0%
SHEET-FORMING COLLAGEN	5	342	7	8941	0.000	0.0%
BASEMENT MEMBRANE	10	342	49	8941	0.000	0.0%
NUCLEUS	125	342	2557	8941	0.001	0.0%
ANCHORING COLLAGEN	4	342	9	8941	0.004	0.0%
INTRACELLULAR	228	342	5326	8941	0.004	0.0%
COLLAGEN TYPE V	3	342	3	8941	0.004	0.0%
COLLAGEN TYPE I	3	342	3	8941	0.004	0.0%
EXTRACELLULAR REGION	54	342	973	8941	0.004	0.0%

Appendix 7-15. GO Biological process terms enriched among miR-29 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
PHOSPHATE TRANSPORT	20	355	68	9408	0.000	0.0%
DEVELOPMENT	104	355	1598	9408	0.000	0.0%
ANION TRANSPORT	23	355	151	9408	0.000	0.0%
CELL ADHESION	43	355	548	9408	0.000	0.0%
REGULATION OF CELLULAR METABOLISM	88	355	1599	9408	0.000	0.0%
REGULATION OF TRANSCRIPTION	82	355	1472	9408	0.000	0.0%
REGULATION OF NUCLEIC ACID METABOLISM	83	355	1495	9408	0.000	0.0%
ION TRANSPORT	39	355	597	9408	0.001	0.0%
TRANSCRIPTION, DNA-DEPENDENT	76	355	1435	9408	0.001	0.0%
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE SIGNALING PATHWAY	12	355	117	9408	0.004	0.0%
ENZYME LINKED RECEPTOR PROTEIN SIGNALING PATHWAY	14	355	156	9408	0.006	0.0%
SKELETAL DEVELOPMENT	12	355	122	9408	0.006	0.0%
DNA MODIFICATION	4	355	15	9408	0.017	6.5%
TRANSCRIPTION FROM RNA POLYMERASE II PROMOTER	26	355	423	9408	0.017	6.7%
INSULIN RECEPTOR SIGNALING	4	355	18	9408	0.028	10.5%

Appendix 7-16. GO Molecular function terms enriched among miR-133 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
PHOSPHOPROTEIN PHOSPHATASE ACTIVITY	9	132	128	9167	0.000	0.0%
TRANSCRIPTION REGULATOR ACTIVITY	27	132	1002	9167	0.002	0.0%
PROTEIN BINDING	66	132	3398	9167	0.002	0.0%
PHOSPHORIC ESTER HYDROLASE ACTIVITY	11	132	238	9167	0.002	0.0%
PRENYLATED PROTEIN TYROSINE PHOSPHATASE ACTIVITY	5	132	61	9167	0.011	0.0%
CALCIUM-DEPENDENT PROTEIN SERINE/THREONINE PHOSPHATASE ACTIVITY	4	132	41	9167	0.020	7.1%
PROTEIN PHOSPHATASE TYPE 1 ACTIVITY	4	132	40	9167	0.019	7.7%
PROTEIN PHOSPHATASE TYPE 2B ACTIVITY	4	132	39	9167	0.018	11.1%

Appendix 7-17. GO Cellular component terms enriched among miR-133 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
INTRACELLULAR ORGANELLE	83	121	4330	8448	0.000	0.0%
MEMBRANE-BOUND ORGANELLE	74	121	3740	8448	0.000	0.0%
NUCLEUS	54	121	2482	8448	0.000	0.0%
INTRACELLULAR	92	121	5164	8448	0.000	0.0%
ORGANELLE ENVELOPE	9	121	191	8448	0.006	0.0%
ENVELOPE	9	121	194	8448	0.006	0.0%
PROTEIN PHOSPHATASE TYPE 2A COMPLEX	3	121	15	8448	0.019	10.0%

Appendix 7-18. GO Biological process terms enriched among miR-133 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
REGULATION OF PHYSIOLOGICAL PROCESS	53	130	2337	8892	0.000	0.0%
PROTEIN AMINO ACID DEPHOSPHORYLATION	8	130	110	8892	0.001	0.0%
REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	36	130	1447	8892	0.001	0.0%
DEPHOSPHORYLATION	8	130	114	8892	0.001	0.0%
REGULATION OF TRANSCRIPTION	35	130	1424	8892	0.002	0.0%
REGULATION OF CELLULAR METABOLISM	37	130	1551	8892	0.002	0.0%
PROTEIN LOCALIZATION	15	130	412	8892	0.003	0.0%
PROTEIN TRANSPORT	14	130	388	8892	0.004	0.0%

Appendix 7-19. GO Molecular function terms enriched among miR-143 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
TRANSFERASE ACTIVITY, TRANSFERRING PHOSPHORUS-CONTAINING GROUPS	19	126	688	9743	0.003	0.0%
EPIDERMAL GROWTH FACTOR RECEPTOR ACTIVITY	3	126	7	9743	0.003	0.0%
KINASE ACTIVITY	17	126	592	9743	0.004	0.0%
TRANSFERASE ACTIVITY	26	126	1120	9743	0.004	0.0%
BINDING	104	126	7066	9743	0.007	0.0%
PROTEIN KINASE ACTIVITY	13	126	430	9743	0.009	0.0%
TRANSCRIPTIONAL ACTIVATOR ACTIVITY	8	126	191	9743	0.011	0.0%
PHOSPHOTRANSFERASE ACTIVITY, ALCOHOL GROUP AS ACCEPTOR	14	126	503	9743	0.012	0.0%
INSULIN-LIKE GROWTH FACTOR BINDING	3	126	19	9743	0.024	6.7%
TRANSCRIPTION REGULATOR ACTIVITY	22	126	1033	9743	0.022	7.1%
PROTEIN-TYROSINE KINASE ACTIVITY	7	126	168	9743	0.021	7.7%

Appendix 7-20. GO Cellular component terms enriched among miR-143 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
INTRINSIC TO PLASMA MEMBRANE	20	116	1124	8941	0.128	210%
NON-MEMBRANE-BOUND ORGANELLE	19	116	1078	8941	0.148	217%
PLASMA MEMBRANE	26	116	1545	8941	0.127	233%

Appendix 7-21. GO Biological process terms enriched among miR-143 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
DEVELOPMENT	36	121	1598	9408	0.001	0.0%
MORPHOGENESIS	16	121	517	9408	0.002	0.0%
TRANSCRIPTION	31	121	1534	9408	0.009	0.0%
POSITIVE REGULATION OF TRANSCRIPTION, DNA-DEPENDENT	5	121	67	9408	0.010	0.0%

Appendix 7-22. GO Molecular function terms enriched among miR-195/424 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
BINDING	335	397	7066	9743	0.000	0.0%
PROTEIN BINDING	195	397	3453	9743	0.000	0.0%
PROTEIN KINASE ACTIVITY	43	397	430	9743	0.000	0.0%
PROTEIN SERINE/THREONINE KINASE ACTIVITY	31	397	306	9743	0.000	0.0%
TRANSFERASE ACTIVITY, TRANSFERRING PHOSPHORUS-CONTAINING GROUPS	51	397	688	9743	0.000	0.0%
CAMP-DEPENDENT PROTEIN KINASE ACTIVITY	19	397	172	9743	0.000	0.0%
PROTEIN-TYROSINE KINASE ACTIVITY	17	397	168	9743	0.001	0.0%
THIOLESTER HYDROLASE ACTIVITY	7	397	39	9743	0.005	0.0%
TRANSCRIPTION REGULATOR ACTIVITY	59	397	1033	9743	0.006	0.0%
CAMP-DEPENDENT PROTEIN KINASE REGULATOR ACTIVITY	4	397	10	9743	0.006	0.0%
MICROTUBULE MOTOR ACTIVITY	8	397	56	9743	0.007	0.0%
PROTEIN KINASE REGULATOR ACTIVITY	7	397	48	9743	0.013	0.0%
INSULIN RECEPTOR SUBSTRATE BINDING	3	397	4	9743	0.009	0.0%
PROTEIN DOMAIN SPECIFIC BINDING	7	397	45	9743	0.009	3.9%
UBIQUITIN-SPECIFIC PROTEASE ACTIVITY	6	397	32	9743	0.009	4.4%
PHOSPHOPROTEIN PHOSPHATASE ACTIVITY	12	397	131	9743	0.018	4.6%

Appendix 7-23. GO Cellular component terms enriched among miR-195/424 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
GOLGI-ASSOCIATED VESICLE	8	373	41	8941	0.001	0.0%
CYTOPLASMIC MEMBRANE-BOUND VESICLE	14	373	126	8941	0.002	0.0%
CAMP-DEPENDENT PROTEIN KINASE COMPLEX	4	373	8	8941	0.003	0.0%
MICROTUBULE ASSOCIATED COMPLEX	10	373	78	8941	0.005	0.0%
TRANSPORT VESICLE	6	373	28	8941	0.005	0.0%
INTEGRAL TO PLASMA MEMBRANE	64	373	1120	8941	0.007	0.0%
COATED VESICLE	10	373	89	8941	0.011	8.3%
TRANS-GOLGI NETWORK TRANSPORT VESICLE	5	373	20	8941	0.008	9.1%

Appendix 7-24. GO Biological process terms enriched among miR-195/424 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
PROTEIN MODIFICATION	80	395	1036	9408	0.000	0.0%
PROTEIN AMINO ACID PHOSPHORYLATION	40	395	427	9408	0.000	0.0%
CELL ADHESION	46	395	548	9408	0.000	0.0%
CELL CYCLE	45	395	559	9408	0.000	0.0%
VESICLE-MEDIATED TRANSPORT	27	395	267	9408	0.000	0.0%
CELL ORGANIZATION AND BIOGENESIS	69	395	1146	9408	0.002	0.0%
CELLULAR PROTEIN METABOLISM	105	395	1910	9408	0.002	0.0%
ENZYME LINKED RECEPTOR PROTEIN SIGNALING PATHWAY	16	395	156	9408	0.002	0.0%
REGULATION OF PROTEIN KINASE ACTIVITY	13	395	113	9408	0.003	0.0%
WNT RECEPTOR SIGNALING PATHWAY	11	395	85	9408	0.003	0.0%
UBIQUITIN CYCLE	21	395	256	9408	0.005	0.0%
UBIQUITIN-DEPENDENT PROTEIN CATABOLISM	10	395	83	9408	0.008	0.0%
MODIFICATION-DEPENDENT PROTEIN CATABOLISM	10	395	83	9408	0.008	0.0%
TRANSMEMBRANE RECEPTOR PROTEIN TYROSINE KINASE SIGNALING PATHWAY	12	395	117	9408	0.010	1.8%
ENDOCYTOSIS	12	395	116	9408	0.009	1.9%
INTRACELLULAR RECEPTOR-MEDIATED SIGNALING PATHWAY	6	395	31	9408	0.009	1.9%
DEPHOSPHORYLATION	12	395	115	9408	0.009	2.0%
MAPKKK CASCADE	9	395	78	9408	0.016	3.3%
CELL DIFFERENTIATION	29	395	431	9408	0.014	3.5%
CELLULAR PROTEIN CATABOLISM	11	395	112	9408	0.019	4.6%
PROTEOLYSIS DURING CELLULAR PROTEIN CATABOLISM	11	395	111	9408	0.018	4.8%
INSULIN RECEPTOR SIGNALING PATHWAY	4	395	18	9408	0.037	9.4%
REGULATION OF TRANSCRIPTION	76	395	1472	9408	0.037	9.5%

Appendix 7-25. GO Molecular function terms enriched among miR-208 targets.

GO Molecular function term	List hits	List total	Pop hits	Pop total	EASE score	FDR
BINDING	85	96	7066	9743	0.000	0.0%
NUCLEIC ACID BINDING	36	96	2056	9743	0.000	0.0%
RNA BINDING	11	96	380	9743	0.004	0.0%
TRANSCRIPTION FACTOR ACTIVITY	16	96	764	9743	0.007	0.0%
PROTEIN KINASE ACTIVITY	11	96	430	9743	0.009	0.0%
PHOSPHOPROTEIN PHOSPHATASE ACTIVITY	6	96	131	9743	0.009	0.0%
DNA BINDING	25	96	1539	9743	0.011	0.0%
PROTEIN SERINE/THREONINE PHOSPHATASE ACTIVITY	4	96	56	9743	0.017	0.0%
PROTEIN SERINE/THREONINE KINASE ACTIVITY	8	96	306	9743	0.030	5.9%
CALMODULIN BINDING	5	96	119	9743	0.029	6.3%

Appendix 7-26. GO Cellular component terms enriched among miR-208 targets.

GO Cellular component term	List hits	List total	Pop hits	Pop total	EASE score	FDR
NUCLEUS	47	83	2557	8941	0.000	0.0%
MEMBRANE-BOUND ORGANELLE	50	83	3850	8941	0.002	0.0%
INTRACELLULAR	60	83	5326	8941	0.013	0.0%
INTRACELLULAR ORGANELLE	52	83	4461	8941	0.016	0.0%
CALCINEURIN COMPLEX	2	83	4	8941	0.036	28.6%

Appendix 7-27. GO Biological process terms enriched among miR-208 targets.

GO Biological process term	List hits	List total	Pop hits	Pop total	EASE score	FDR
DEVELOPMENT	32	94	1598	9408	0.000	0.0%
TRANSCRIPTION	28	94	1534	9408	0.001	0.0%
REGULATION OF TRANSLATIONAL INITIATION	4	94	27	9408	0.002	0.0%
RNA PROCESSING	9	94	237	9408	0.002	0.0%
CELLULAR METABOLISM	62	94	4903	9408	0.006	0.0%
REGULATION OF TRANSCRIPTION	25	94	1472	9408	0.008	0.0%
RNA METABOLISM	9	94	291	9408	0.008	0.0%
TRANSLATIONAL INITIATION	4	94	42	9408	0.008	0.0%
WNT RECEPTOR SIGNALING PATHWAY	5	94	85	9408	0.010	0.0%
SKELETAL MUSCLE DEVELOPMENT	3	94	18	9408	0.013	0.0%
MUSCLE DEVELOPMENT	5	94	95	9408	0.014	0.0%
PROTEIN POLYUBIQUITINATION	2	94	3	9408	0.029	2.7%
RNA SPLICING	5	94	116	9408	0.028	2.8%
PROTEIN AMINO ACID DEPHOSPHORYLATION	5	94	111	9408	0.024	3.0%
CELL PROLIFERATION	10	94	473	9408	0.044	7.9%
CELL DIFFERENTIATION	9	94	431	9408	0.063	10.9%

Appendix 7-28. RNA concentrations from myotubes treated with 100 nM or 0.1 nM insulin used in Chapter 5. RNA quantification conducted using the Nanodrop spectrophotometer. A 260/230 ratio of <1.5 indicates possible phenol contamination during RNA isolation.

Sample ID	Insulin	Time	RNA ng/µl	260/280	260/230
HGDM41	0 nM	0 min	559.33	1.96	2.06
HGDM42	0 nM	0 min	868.95	2.12	2.17
HGDM43	0 nM	0 min	860.9	1.99	2.13
HGI1	100 nM	10 min	630.19	1.9	2.39
HGI2	100 nM	10 min	620.02	1.9	2.28
HGI3	100 nM	10 min	548.1	1.92	2.23
HGI4	0.1 nM	10 min	510.81	1.96	2.19
HGI5	0.1 nM	10 min	633.08	1.9	2.45
HGI6	0.1 nM	10 min	377.92	1.79	2.11
HGI7	100 nM	30 min	680.09	1.87	2.19
HGI8	100 nM	30 min	697.42	1.87	2.15
HGI9	100 nM	30 min	519.25	1.89	2.34
HGI10	0.1 nM	30 min	660.83	1.89	2.22
HGI11	0.1 nM	30 min	579.96	1.86	2.37
HGI12	0.1 nM	30 min	909.59	1.97	2.18
HGI13	100 nM	60 min	386.6	1.92	2.4
HGI14	100 nM	60 min	420.51	1.89	2.44
HGI15	100 nM	60 min	338.9	1.94	2.56
HGI16	0.1 nM	60 min	512.31	1.94	2.53
HGI17	0.1 nM	60 min	370.1	1.9	2.46
HGI18	0.1 nM	60 min	389.22	1.88	2.46
HGI19	0 nM	60 min	302.44	1.96	2.66
HGI20	0 nM	60 min	252.69	1.91	2.65
HGI21	0 nM	60 min	536.44	1.93	2.47

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